The molecular biology of Naegleria fowleri

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THE MOLECULAR BIOLOGY OF *NAEGLERIA FOWLERI*

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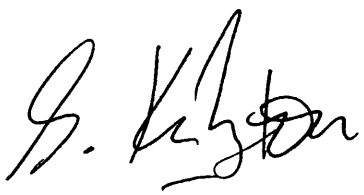

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SUMMARY

Naegleria fowleri is a small free-living amoeba found in thermal aquatic sites worldwide. The organism is pathogenic to man causing fatal primary amoebic meningoencephalitis (PAM). Infection results from the intranasal inoculation of the organism usually whilst bathing. In 1978 a fatal case of PAM occurred in Bath Spa, England. Infection was traced to a public swimming pool fed with water from the natural hot springs that rise in the City at the Roman Baths complex. When monitoring the environment for the presence of *N. fowleri* it is important to reliably differentiate the organism from other closely related but nonpathogenic thermophilic species. This can be achieved by either animal pathogenicity testing, biochemical analysis using isoenzyme electrophoresis or detection of whole-cell DNA restriction fragment length polymorphisms (RFLPs). Several disadvantages are encountered with these methods. Animal pathogenicity tests are expensive, time consuming and increasingly socially unacceptable. Biochemical and molecular studies, whilst effective in differentiating the species, are laborious and expensive to perform and require large numbers of trophozoites which usually have to be adapted to growth in semi-defined broth media. Accordingly, these methods do not lend themselves to the examination of large numbers of isolates frequently encountered from environments such as the thermal springs of Bath.

To this end it was decided to investigate the potential of molecular biological techniques in the development of rapid and reliable methods for the identification of *N. fowleri* from the environment. In the first instance a simple isoenzyme test for glucose phosphate isomerase using cellulose acetate membrane electrophoresis was developed. This enabled the differentiation of *N. fowleri* from other thermophilic *Naegleria* on the basis of the relative mobility of a single enzyme band. The test was, however, limited by the need to subculture strains following primary isolation and also by the number of samples which could be analyzed on each membrane. DNA probes were therefore produced for use in the detection of *N. fowleri* directly following primary culture isolation. A genomic library of *N. fowleri* DNA was constructed in the λ phage vector EMBL3 and subclones made into the plasmid vector pUC 18. After rigorous screening, two clones, pB2.3 (a 1.2 kbp insert) and pB2.2.4 (a 0.8 kbp insert) were shown to be specific in their hybridisation to *N. fowleri* DNA and in titration experiments could detect as little as 6.25 pg of DNA, equivalent to about 36 trophozoites. A third pUC 18 subclone pB2.2 (a 6.1 kbp insert) was found to contain a repeated DNA element that enabled the detection of chromosomal restriction fragment length polymorphisms (RFLPs) which demonstrated differences between strains of *N. fowleri* from different geographic localities.

Clone pB2.3 was partially sequenced and oligonucleotide primers synthesised for use in a polymerase chain reaction (PCR) assay for the organism. After rigorous evaluation, the test was shown to amplify only *N. fowleri* DNA and could detect 10 *N. fowleri* trophozoites or cysts after 35 PCR cycles or as little as 1 organism after 45 cycles. Both the DNA probes and the PCR were used to develop protocols that facilitated the simple and rapid identification of *N. fowleri* from the thermal springs of Bath.

In a separate environmental survey, *N. fowleri* was isolated from the cooling circuits of two electricity power stations in Nottingham, England and the River Trent supplying them. Detection of RFLPs both directly on agarose gel electrophoresis and in conjunction with the repetitive element DNA clone pB2.2 showed these strains to differ from those present in the thermal springs of Bath but they did resemble certain *N. fowleri* isolates from a nuclear power station in France. This RFLP type was not found in any other *N. fowleri* strains from various geographic localities worldwide. Prior to this study the only isolates of *N. fowleri* in the United Kingdom had come from the Bath PAM case and the thermal springs that were the source of the infection. Accordingly, these represent only the second such isolates of *N. fowleri* to be made in the United Kingdom and indicate that the organism may be more widely distributed in this country than has previously been supposed.

LIST OF ABBREVIATIONS

~ ..... approximately
λ ..... lambda
AIDS ..... acquired immunodeficiency syndrome
bp ..... base pairs
BSA ..... bovine serum albumin
cm ..... centimetre
CSF ..... cerebro spinal fluid
DEPC ..... diethylpyrocarbonate
dH_2O ..... distilled water
ddH_2O ..... double-distilled water
DMSO ..... dimethylsulphoxide
DNA ..... deoxyribonucleic acid
DNase ..... deoxyribonuclease
EBr ..... ethidium bromide
EDTA(Na_2) ..... ethylenediamine tetraacetic acid (disodium)
fg ..... femtogram
FLA ..... free-living amoeba(e)
g ..... gram
GAE ..... granulomatous amoebic meningoencephalitis
HCl ..... hydrochloric acid
IPTG ..... isopropylthio-β-D-galactoside
ITS ..... internal transcribed spacers
kbp ..... kilo base pairs
M ..... molar
mA ..... milliamps
mbp ..... mega base pair
mg ..... milligram
ml ..... millilitre
mm ..... millimetre
mM ..... millimolar
mtDNA ..... mitochondrial DNA
MTT ..... (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl
N ..... normal solution
NADP ..... nicotinamide adenine dinucleotide phosphate
NaOH ..... sodium hydroxide
ng ..... nanogram
NNA-*E. coli* ..... non-nutrient agar seeded with *Escherichia coli*

°C . . . . . degrees centigrade
OD . . . . . optical density
OTU's . . . . . operational taxonomic units
PAM . . . . . primary amoebic meningoencephalitis
PBS . . . . . phosphate buffered saline
PCR . . . . . polymerase chain reaction
PFU . . . . . plaque forming units
pg . . . . . picogram
PMS . . . . . phenazine methasulphate
pUC . . . . . type of plasmid cloning vector
rDNA . . . . . ribosomal deoxyribonucleic acid
RFLPs . . . . . restriction fragment length polymorphisms
RNA . . . . . ribonucleic acid
RNase . . . . . ribonuclease
rRNA . . . . . ribosomal ribonucleic acid
rpm . . . . . revolutions per minute
SCGYM . . . . . serum-casein-glucose-yeast extract medium
#SCGYM . . . . . SCGYM supplemented with 0.1% Panmede liver digest
SDS . . . . . sodium dodecyl sulphate
sp . . . . . species (singular)
spp . . . . . species (plural)
ssDNA . . . . . single stranded deoxyribonucleic acid
SSU . . . . . small subunit
TAE . . . . . tris-acetate-EDTA
TBE . . . . . tris-borate-EDTA
Tris . . . . . tris(hydroxymethyl)aminomethane
U . . . . . units
$\mu\ell$ . . . . . microlitre
UV . . . . . ultra violet
v/cm . . . . . volts per centimetre
v/v . . . . . volume per volume
w/v . . . . . weight per volume
x g . . . . . times gravity
X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactoside
YPNFH . . . . . yeast extract-peptone-yeast nucleic acid-folic acid-haemin
#YPNFH . . . . . YPNFH supplemented with 0.1% glucose and 10% foetal calf serum

1. THE MOLECULAR BIOLOGY OF *NAEGLERIA FOWLERI*

1.1. INTRODUCTION

Free-living amoebae (FLA) are unicellular protozoa characterised by a feeding and replicating trophozoite which, in many species, can produce a dormant cyst stage (Page, 1988). In certain genera, the trophozoites can also transform into highly motile flagellates (Page, 1988). FLA are found in soil and aquatic environments, feeding on bacteria, algae, fungi and other protists (Singh, 1975; Singh *et al*, 1984; Page, 1988). The resistance of the cysts in most species to extremes of temperature (Biddick *et al*, 1984; Kilvington, 1989), disinfection (Chang, 1978; Kilvington, 1990) and desiccation (Kingston & Warhurst, 1969) accounts for the almost ubiquitous distribution of these organisms in nature. Accordingly, FLA have been isolated from almost any environmental site that has been cared to be examined including chlorinated waters (De Jonckheere, 1979; Esterman *et al*, 1984a), dust (Rivera *et al*, 1987), antarctic tundra (Brown *et al*, 1982) and the atmosphere (Kingston & Warhurst, 1969, Rivera *et al*, 1987).

The study of FLA stems from the development of the first primitive microscopes in the 17th century by Antony van Leeuwenhoek (1632-1723) who observed a variety of microbes, including protozoa, for the first time. Although FLA were undoubtedly seen, these were not recorded in the detailed descriptions and drawings of the "animalcules" left by Leeuwenhoek. It was Von Rosenhof (1755) who first observed a FLA. The large single motile cell was seen in a sample of pond water and was called "small-proteus". Later, Linnaeus classified it as one of the giant amoebae known today as *Chaos proteus* (Page, 1988). Dujardin (1841) first described a small FLA, *Amoeba limax* and later made a detailed study of the variety of FLA present in aquatic sediments. As the optical resolution of the microscopes improved and the interest in protozoa grew, many new types of FLA were reported (Schardinger, 1899). Such studies were later aided by the demonstration that FLA could be cultured on plain agar overlaid with a bacterial food source (Nakamura, 1951; Singh, 1975). This provided biologists with the means for studying the environmental distribution of FLA, their role in soil ecology and their locomotive and feeding behaviour (Singh, 1975; Singh *et al*, 1984).

Although FLA had frequently been isolated from the faeces of humans and animals (Walker, 1908), the only amoeba considered to be pathogenic for man was *Entamoeba histolytica*, the anaerobic parasite causing amoebic dysentery (Schaudinn, 1903; Gitler & Mirelman, 1986)). The first indication that FLA may have pathogenic potential came in 1957 when Jahnes reported the cytopathogenicity of an amoeba, identified as an *Acanthamoeba*, that contaminated monkey kidney tissue culture cell lines (Jahnes, *et al*, 1957). The following year Clyde Culbertson, working on the production of polio virus vaccine, observed similar *Acanthamoeba* contamination of tissue culture cell lines. Inoculation of the *Acanthamoeba*, either intracerebrally or intranasally into monkeys, rabbits or mice resulted in fatal meningoencephalitis (Culbertson *et al*, 1958 & 1959; Culbertson, 1971). *Acanthamoeba* were reisolated from the brain and lungs of moribund animals and were observed in post mortem sections of infected tissue.

In describing these findings, Culbertson speculated on the potential of *Acanthamoeba*, and possibly other FLA, to produce similar disease in man. This was realised in 1965 with the description by Fowler and Carter (1965) of four cases of amoebic meningoencephalitis in South Australia. Because the amoebae were only identified from preserved post mortem specimens, the infecting organism was considered to be an *Acanthamoeba* after Culbertson's earlier findings. Similar cases, also identified post mortem, were reported from the USA around this period, with Butt (1966) naming the disease primary amoebic meningoencephalitis or PAM.

1.2. *Naegleria fowleri* primary amoebic meningoencephalitis (PAM)

It was not until 1969 that Carter in Australia isolated an amoeba from a freshly taken specimen of cerebro spinal fluid (CSF) of a PAM case. The organism did not, however, resemble an *Acanthamoeba* but was similar in morphology and movement to FLA of the genus *Naegleria* (Carter, 1969 & 1970; Page, 1988). Around this time, workers in the USA made similar clinical isolates and showed that the trophozoites could transform into a temporary, highly motile flagellate form when placed into distilled water (Nelson & Jones, 1970). This feature is characteristic of the genus *Naegleria* and in 1970 Carter proposed the new species *N. fowleri* for the causative agent of PAM (Carter, 1970). PAM results from the intranasal inoculation of the organism (most likely in the trophozoite form) usually whilst bathing (Carter, 1972; John, 1982; Warhurst, 1985; John, 1993). From the anterior nares the organism infects the nasal epithelium and migrates along the olfactory lobes, via the cribriform plate to infect the brain and meninges (Carter, 1972; Warhurst, 1985). Clinical signs and appearance of the CSF are typical of acute pyogenic meningitis (Carter, 1972; Martinez, 1985 & 1987). PAM is almost invariably fatal, with death occurring 3-10 days after exposure (John, 1993; Warhurst, 1985; Martinez, 1987). Infection occurs most frequently in young males, probably because of their more vigorous bathing habits (John, 1982).

1.2.1. Treatment of PAM

Only five patients have survived PAM, as described from cases in Australia (Anderson & Jamieson, 1972; Dorsch *et al*, 1983), England, (Apley *et al*, 1970), Mexico (Rodriguez Perez, 1984) and the USA (Seidel *et al*, 1982; Brown, 1991). In each case the antifungal agent amphotericin B was used in the treatment. Amphotericin B is a polyene compound and lipophilic, binding to the trophozoite cell membrane causing leakage of the cytoplasmic contents (Carter, 1969; Duma & Finley, 1976; Kobayashi & Medoff, 1977; Thong *et al*, 1979; Stevens *et al*, 1981). Although *N. fowleri* is highly sensitive *in vitro* to amphotericin B, the drug does not readily traverse the blood-brain barrier. This feature, together with the rapid progress of the infection and usually delayed diagnosis, accounts for the poor prognosis in PAM. Furthermore, it has been suggested that trophozoite lysis and accompanying release of intracellular cytotoxic compounds by the amphotericin B therapy only adds to the severity of the infection (Ferrante, 1986).

1.2.2. Epidemiology of PAM

Approximately 200 cases of PAM have been reported worldwide since 1965 (Martinez, 1985; Visvesvara & Stehr-Green, 1990) including: Australia (Carter, 1972, Dorsch *et al*, 1983), Belgium (Jadin *et al*, 1971), Czechoslovakia (Cerva & Novak, 1968; Kadlec *et al*, 1978), India (Martinez, 1985), Mexico (Lares-Villa *et al*, 1993), New Guinea (Martinez, 1985), New Zealand (Cursons *et al*, 1979), Nigeria (Lawande, 1980), the United Kingdom (Symmers, 1969; Apley *et al*, 1970; Cain *et al*, 1981), USA (Butt, 1966, Dos Santos, 1970; Stevens *et al*, 1981; Seidel *et al*, 1982), Thailand (Charoenlarp *et al*, 1989) and Venezuela (Moreno *et al*, 1990).

Clustering of PAM cases can occur when a common focus is the source for infection. In Usti, Czechoslovakia 16 cases of PAM occurred in a 4 year period from 1962 to 1965. In all cases the victims had swum in a public bathing pool, although repeated attempts to isolate *N. fowleri* from the water failed to yield the organism (Cerva & Novak, 1968). It was eventually realised that at sometime the length of the pool had been shortened by the construction of a false panel at one end. Examination of stagnant water that had accumulated behind the partition wall eventually yielded isolates of *N. fowleri* (Kadlec *et al*, 1978).

In South Western Australia infections were associated with the reticulated mains water supply (Dorsch *et al*, 1983). In this region, water is supplied to remote localities via overground steel pipes. Solar heating of the water inactivated the chlorine used for disinfection in the system and enabled *N. fowleri* to proliferate (Esterman *et al*, 1984b). As a result some 20 PAM cases occurred. The trophozoites and cysts of *N. fowleri* are sensitive to at least 1 mg/l free available chlorine (Kilvington, 1990), and the installation of chlorifiers at regular intervals along the pipe lines, together with regular monitoring of the water supply has now eliminated the problem (personal communication, Mr Peter Christy, State Water Laboratories, Salisbury, South Western Australia).

Cases of PAM continue to occur worldwide (Lares-Villa *et al*, 1993) although many undoubtedly pass unrecognised because of incorrect diagnosis, or are no longer considered worth reporting in the literature (Personal communication, Dr Govinda Visvesvara, Centres for Disease Control, Atlanta, Georgia, USA).

1.2.2.1. PAM in the United Kingdom

Cases of PAM from the United Kingdom have been described. Symmers (1969) identified amoebae in post mortem sections of brain material from a young Essex boy who died in 1909. The pathological findings of the case were identical to those described by Carter (1970 & 1972) for PAM. A second case was described from Belfast, Northern Ireland. Whilst a resident medical student in 1937, Symmers observed amoebae with a slug-like movement in a CSF specimen taken from a 10 year old girl who died two hours after admission to hospital. About 10 days previously the child had been rescued from drowning

at a public swimming place in the Belfast City Water Works. The features of this second case almost certainly indicate it to have been PAM.

Apley *et al*, (1970) described a fatal case of PAM in a young child who played with 2 others in a garden puddle in Bristol one summer. Amoebae were isolated from the CSF of the child who subsequently died 16 days after admission to hospital despite intensive therapy with amphotericin B. The other two children showed signs of meningitis, although amoebae were not seen in CSF specimens, and both recovered following amphotericin B therapy. Although the clinical findings and the isolation of an amoeba from the child who died indicated PAM, examination of the organism at a later date showed it not to be *N. fowleri* but rather *N. gruberi* (Saygi *et al*, 1973). This species is nonpathogenic, growing poorly or not at all at 37°C (see *1.8. Taxonomy of the Naegleria*). In view of these unusual findings, the diagnosis of PAM in the Bristol cases must be considered uncertain.

One confirmed case of PAM occurred in Bath Spa, England in 1978. The victim was a young girl who swam in a public bathing pool fed with water from the historic hot springs that rise naturally in the City. Trophozoites of *N. fowleri* were observed in, and subsequently cultured from, a specimen of CSF. Despite intensive therapy with amphotericin B, which eliminated amoebae from subsequent CSF samples, the child died 9 days after admission to hospital (Cain *et al*, 1981). This case and the epidemiology surrounding it are discussed in *1.11. Primary amoebic meningoencephalitis in Bath Spa* later in this chapter.

1.3. *Acanthamoeba* infections of man

From Culbertson's observation on the animal pathogenicity of *Acanthamoeba* (Culbertson *et al*, 1958 & 1959), the first reported case of human infection due to a FLA by Fowler and Carter (1965) was attributed to this organism. However, it is now known that this was caused by *N. fowleri* (Carter, 1969 & 1970). Similarly, Patras and Andujar (1966) described a case of *Acanthamoeba* meningoencephalitis in a patient who died 18 days after admission to hospital in Texas, USA which can be attributed retrospectively to *N. fowleri*.

Kenney (1971) first demonstrated that *Acanthamoeba* could infect man. Using a complement fixation test he showed rising antibody titres to *A. culbertsoni* in a patient who subsequently died of cerebral granulomatous disease. Amoebae resembling *Acanthamoeba* were also observed in post mortem brain sections. The following year, Jager and Stamm (1972) observed trophozoites and cysts in post mortem brain sections of a patient with Hodgkin's disease who had been receiving prolonged immunosuppressive therapy. Significantly, the amoebae reacted positively in an indirect fluorescent antibody test using antiserum to *Acanthamoeba* spp. but not with antiserum to *E. histolytica*, *N. gruberi* or *N. fowleri*.

1.3.1. Acanthamoeba granulomatous amoebic meningoencephalitis

Martinez (1980) first used the termed granulomatous amoebic encephalitis (GAE) to describe *Acanthamoeba* cerebral infection and to distinguish it from *N. fowleri* PAM. At least 50 cases of GAE have been described worldwide with reports from England, India, Korea, Nigeria, Peru, USA, Venezuela and Zambia (Martinez, 1985; De Jonckheere, 1987a; Visvesvara & Stehr-Green, 1990). Patients are usually immunosupressed either from chemotherapy, alcohol abuse or other chronic disease (Martinez, 1985; Martinez & Janitschke, 1985). GAE as the primary cause of death in patients with acquired immunodeficiency syndrome (AIDS) has been reported (Gonzales *et al*, 1986, Wiley *et al*, 1987; Visvesvara & Stehr-Green 1990).

GAE is chronic and invariably fatal. The diagnosis is difficult because, unlike PAM, amoebae are not usually seen in the CSF which microscopically appears consistent with viral encephalitis. Consequently, most cases of GAE are made post mortem. The clinical course of the disease ranges from 8->32 days from the onset of symptoms which include seizures, fever, hemiparesis, headache, meningism and visual abnormalities. The route of infection in GAE is unclear. It has been suggested that invasion of the brain results from haematogenous spread from a primary infection elsewhere in the body (Martinez, 1985; Warhurst, 1985).

1.3.1.1. Treatment of GAE

Because GAE is usually diagnosed post mortem, few cases exist where anti-acanthamoeba therapy has been instigated. Several antimicrobial agents have shown to be active *in vitro* against pathogenic *Acanthamoeba*, including propamidine and pentamidine isethionate, ketoconazole, clotrimazole, neomycin, polymyxin E and paromomycin (Casemore, 1970; Duma & Finley, 1976; Nagington & Richards, 1976; Ferrante *et al*, 1984; Wright *et al*, 1985; Driebe *et al*, 1988; Kilvington *et al*, 1990). Sulphadiazine and rifampicin have been shown to protect animals from experimental infections but it remains to be proven whether these have any effect in cases of human disease (Culbertson *et al*, 1965; Das *et al*, 1991).

1.3.2. *Acanthamoeba* keratitis

Whilst GAE is rare, keratitis (infection of the cornea) due to *Acanthamoeba* occurs with far greater frequency. The disease was first recognised in the USA by Jones and colleagues in a case associated with fatal meningoencephalitis (Jones *et al*, 1975). Nagington also described cases of *Acanthamoeba* keratitis in the United Kingdom at this time (Nagington *et al*, 1974). *Acanthamoeba* keratitis usually affects previously healthy persons and in the untreated state can lead to painful blindness (Cohen *et al*, 1987; Lindquist *et al*, 1988; Moore, 1988). Seddon (1988) gives a personal account of the harrowing nature of the disease.

At least 200 cases of *Acanthamoeba* keratitis have been reported in the USA up to 1989 (Stehr-Green *et al*, 1989) and at least 100 in the United Kingdom (Kilvington & White, 1994). Although *Acanthamoeba* keratitis can arise from trauma to the eye, usually in association with contamination by environmental matter (Sharma *et al*, 1990), contact lens wearers are most at risk from infection and account for approximately 85% of reported cases (Moore *et al*, 1987; Stehr-Green *et al*, 1989). The reasons for the higher risk of infection in this latter group are not fully understood. Poor hygienic practices, notably the preparation of home-made saline rinsing solutions and defaulting on lens disinfection procedures, are recognised risk factors. *Acanthamoeba* are ubiquitous in our environment and are present in domestic storage and mains supply water (S. Kilvington, unpublished observations). *Acanthamoeba* have been shown to contaminate contact lens storage containers (Larkin *et al*, 1990) and adhere to the surface of soft and hard contact lenses (Kilvington & Larkin, 1990). The precise mode of infection is unclear but may arise through minor trauma to surface epithelium from contact lens wear which allows the organism to penetrate the cornea.

1.3.2.1. Treatment of *Acanthamoeba* keratitis

Because the cyst form is resistant to most antimicrobial agents at concentrations achievable in the cornea and tolerated by the ocular surface, the treatment of *Acanthamoeba* keratitis is exceedingly difficult. Prolonged medical therapy with antifungal agents (Driebe *et al*, 1988; Ishibashi *et al*, 1990) or propamidine isethionate may yield a cure (Wright *et al*, 1985) or else control the disease sufficiently to allow corneal grafting a chance of success (Cohen *et al*, 1987, Lindquist *et al*, 1988). Most recently, polyhexamethylene biguanide (PHMB) has shown great potential in the treatment of *Acanthamoeba* keratitis. Topical administration of a 0.02% solution of PHMB was first used by Larkin and colleagues (1992) to cure 5 cases of infection which were unresponsive to conventional therapy based on propamidine isethionate or antifungal agents. As a result of prompt diagnosis and intensive therapy involving PHMB the prognosis in *Acanthamoeba* keratitis has changed dramatically enabling most cases to be cured medically with restored normal vision within a few weeks of treatment commencing (personal communication, Mark Elder, Moorfields Eye Hospital, London).

1.3.3. *Acanthamoeba* infections of other sites

Although infections of the skin and eye by *Acanthamoeba* have been associated with GAE (Jones *et al*, 1975; Martinez, 1980; Martinez & Janitschke, 1985) cases where infection was localised elsewhere in the body, such as the ear and bone have been reported (Lengy *et al*, 1971; Borokovitz *et al*, 1981).

1.4. *Balamuthia mandrillaris* "leptomyxid amoeba"

In 1990, Visvesvara and colleagues (1990) reported fatal encephalitis in man and primates due to a previously unknown type of amoeba. Brain tissue of a mandrill baboon that died of meningoencephalitis

was inoculated on to non-nutrient agar plates seeded with a living suspension of *Escherichia coli* (NNA-*E. coli*) and into African green monkey kidney cell culture. NNA-*E. coli* is the conventional medium for the isolation of *Naegleria*, *Acanthamoeba* and other FLA. No growth occurred on NNA-*E. coli* but an amoeba was isolated in tissue culture after 3 weeks incubation. Although no precise classification of the organism could be made at the time, the amoeba resembled FLA of the genus *Leptomyxa* or *Gephyramoeba* (Page, 1988; Pussard & Pons, 1976) and was described as a leptomyxid amoeba. The trophozoites ranged in size from 15-60 μm, were pleomorphic and highly branched. The cysts ranged from 15-30 μm, were round and doubled walled, with the outer wall being thickened and irregular. Intranasal inoculation of the amoeba into mice caused fatal meningoencephalitis within one week. Rabbit polyclonal antiserum raised against the strain did not react with *Acanthamoeba* spp., *Naegleria* spp., several other species of FLA or *E. histolytica*. Using an indirect fluorescent antibody assay, several cases of presumed *Acanthamoeba* GAE were re-examined and 16 cases of human infection and 2 in a gorilla and a sheep caused by the leptomyxid amoeba were identified.

In a subsequent comparative study of the leptomyxid amoeba with members of the genus *Leptomyxa* and *Gephyramoeba*, Visvesvara and colleagues (1993) found the strain to be unique in its animal pathogenicity, antigenic structure and morphology, and *Balamuthia mandrillaris*, N. G., N. Sp was proposed. A further 19 cases of human encephalitis caused by the organism were also identified. As with *Acanthamoeba* GAE, patients are either immunocompromised or very young or old. The majority of cases of infection have been described from the USA although cases have been identified from Argentina, Australia, Canada, Mexico and Peru (Visvesvara *et al*, 1990 & 1993). The clinical course of the disease in humans ranges from 14 days to 6 months with a mean of 75 days and is invariably fatal. Clinical symptoms and histopathological findings are similar to those seen with GAE. At present there exists no reports of *in vitro* or *in vivo* antimicrobial activity against this organism.

No environmental isolates of *B. mandrillaris* have been reported. Because *B. mandrillaris* will not grow on NNA-*E. coli* (the standard medium for the isolation of FLA) attempts to isolate the organism from the environment will prove exceedingly difficult.

1.5. FLA and legionella

The trophozoites of FLA including *Acanthamoeba* and *Naegleria*, have been shown to support the intracellular replication of *Legionella pneumophila*, the bacterium causing the atypical pneumonia of Legionnaires' disease in man (Rowbotham, 1980; Tyndall & Domingue, 1982; Holden, *et al*, 1984; Newsome *et al*, 1985). In co-culture experiments, membrane bound amoeba vesicles containing large numbers of *L. pneumophila* are frequently observed. It has been suggested that inhalation of such vesicles could provide sufficient bacteria to cause Legionnaires' disease (Rowbotham, 1980). Furthermore, *Acanthamoeba* cysts can also afford the bacterium protection from chlorine disinfection at a concentration of at least 50 mg/L (Kilvington & Price, 1990). As *L. pneumophila* is also commonly found in soil and

aquatic sites, it is possible that *Acanthamoeba* and other FLA may have an important role to play in the presence, persistence and colonisation of aquatic systems by this organism.

FLA may also be involved in allergic respiratory complaints termed Humidifier fever or Sick-Building Syndrome. This condition is associated with air-conditioned environments. Symptoms include respiratory complaints, fatigue and general malaise (Anon, 1977). Elevated antibody levels to a variety of microbes, including *Naegleria* and *Acanthamoeba*, have been demonstrated in affected persons (Edwards *et al*, 1976; Anon, 1977; Forsgren *et al*, 1984). It is thought that symptoms arise following a primary sensitisation to microbes released from contaminated air-conditioning units during operation. If such a unit is not used for a time, the microbes proliferate to high numbers and are then released into the environment when the system is next operated thus giving rise to an acute allergic response in previously sensitised persons.

1.6. Ecology of *N. fowleri*

N. fowleri is found in warm freshwater habitats worldwide and has been isolated from natural hot springs (Brown *et al*, 1983; Kilvington *et al*, 1991b), fresh water lakes (Wellings *et al*, 1977; Akao *et al*, 1984; Tyndall *et al*, 1989) domestic water supplies (Anderson & Jamieson, 1972; Esterman *et al*, 1984b), chlorinated swimming pools (Kadlec *et al*, 1978; Cursons *et al*, 1979; Gogate & Deodhar, 1985), thermal discharges from industrial processes (De Jonckheere *et al*, 1975; Willaert & Stevens, 1976; De Jonckheere & Van de Voorde, 1977a; De Jonckheere, 1978; Cerva *et al*, 1980; Sykora *et al*, 1983). In addition, *N. fowleri* has been isolated the cooling circuits and discharge water of electricity power stations plants (Dive *et al*, 1981; Dive *et al*, 1982; Cerva & Simanoz, 1983; Tyndall *et al*, 1989; Huizinga & McLaughlin, 1990).

N. fowleri can tolerate growth between 28°C and 45°C (Griffin, 1972), although is more likely to be isolated from waters where the temperature is above 30°C (De Jonckheere *et al*, 1975). The cysts of *N. fowleri* have been shown to survive at 4°C for at least 8 months with retention of virulence by the excysted trophozoites (Warhurst *et al*, 1980). This may account for cases of PAM from natural waters which are solar heated in the summer months but then freeze during the winter such as the lakes of Virginia, USA (Dos Santos, 1970).

Factors which may account for the presence of *N. fowleri* in a particular environment are largely unknown. Certainly, the habitat must comprise warm freshwater with a plentiful bacterial and other microbial food supply. However, such conditions apply equally to the presence of *N. lovaniensis* and *N. australiensis* which tend to predominate in such environments. Under laboratory conditions, these species grow at a faster rate than *N. fowleri*, suggesting that they may compete unfavourably in the natural environment and thus suppressing its presence. Griffin (1983) proposed the flagellate-empty hypothesis to account for the sporadic environmental distribution of *N. fowleri*. In summary, the hypothesis states that when any human and/or natural events remove amoebae that usually compete successfully against *N.*

fowleri, an advantage is conferred on *N. fowleri* in recolonising the habitat. The trophozoite is transformed into a motile flagellate, which enhances recolonization. Accordingly, if a warm water habitat is cleared repeatedly, this is particularly favourable to *N. fowleri* which may flourish and so give rise to cases of PAM. Detterline and Wilhelm (1991) found evidence to support the hypothesis by showing that the presence of *N. fowleri* in thermal aquatic sites in the USA was statistically significant in recently disturbed environments. However, such changes were also true for the occurrence of *N. lovaniensis* and *Acanthamoeba*.

1.7. Taxonomy of FLA

FLA belong to the kingdom Protista, subkingdom Protozoa, phylum Sarcomastigophora, subphylum Sarcodina, superclass Rhizopodea, class Lobosea, subclass Gymnamoebia. Page (1988) recognised 7 orders of FLA containing 21 families, 49 genera and over 200 species. The *Acanthamoeba* are assigned to the order Acanthapodia, family Acanthamoebidae, and the *Naegleria* to the order Schizopyrenidia, family Vahlkampfiidae. The present classification of the FLA is derived mainly from morphological observations of the life forms of the organisms. The exception are members of the genera *Acanthamoeba* and *Naegleria*, which, because of the pathogenic potential of certain species for man has necessitated the development of reliable identification methods. On the basis of trophozoite and cyst morphology Pussard and Pons (1977) recognised 18 species of *Acanthamoeba* which were assigned to 3 morphological groups. However, the wide variation in cyst morphology that is seen even within clonal populations of species strains has questioned the reliability of this approach to the taxonomic classification of *Acanthamoeba* spp. This has been supported by recent studies using isoenzyme electrophoresis (De Jonckheere, 1983; Daggett *et al*, 1985) and restriction endonuclease analysis of mitochondrial DNA (mtDNA) (Bogler *et al*, 1983; Kilvington *et al*, 1991a; Yagita & Endo, 1991) which have demonstrated large heterogeneity within strains assigned to the same species by morphological criteria as well as homogeneity between different species. Whilst these studies have highlighted the inadequacies of the present identification of *Acanthamoeba* species, there has been no concerted attempt to apply these methods in the redefinition of the genus.

1.8. Taxonomy of the *Naegleria*

The genus *Naegleria* contains 6 species and 2 subspecies which are morphologically identical by light microscopy. With the exception of *N. gruberi*, the species are well defined, being distinguished in part by the maximum temperature tolerated for growth (Griffin, 1972; Page, 1988) and completely by antigenic (Willaert, 1977), isoenzyme (De Jonckheere, 1982a; Pernin, 1984) and DNA analysis (De Jonckheere, 1987c; Clarke *et al*, 1989; Milligan & Band, 1988).

N. gruberi (Schardinger, 1899). Until 1970, *N. gruberi* was the only member of the genus. The species is the most common *Naegleria* found in temperate soil and aquatic sites (Page, 1988). *N. gruberi* is nonpathogenic, growing poorly or not at all at 37°C. Recent studies using isoenzyme and molecular DNA

analysis have shown that the species is a heterogeneous group resulting from the arbitrary allocation of strains to the species because of a temperature tolerance of $\leq 37°C$ (De Jonckheere, 1987c; Clark *et al*, 1989; Pernin & Cariou, 1989; Robinson *et al*, 1992).

N. fowleri (Carter, 1970). In 1970 Carter described *N. fowleri* as the cause of PAM in man. The species is characterised by its pathogenicity for man and laboratory animals and its ability to grow at temperatures up to 45°C (De Jonckheere, 1987b).

N. jadini (Willaert & Le Ray, 1973). *N. jadini* was first isolated from a swimming pool in Belgium. *N. jadini* will not grow at 37° and is nonpathogenic. Using immunoelectrophoresis the strain was shown to be antigenically distinct from *N. fowleri* and strains of *N. gruberi*. Only the original strain of *N. jadini* has ever been reported, others may exist but may have been assigned to the *N. gruberi* complex.

N. lovaniensis (Stevens *et al*, 1980). During the examination of thermal environments for the presence of *N. fowleri* several workers reported isolation of strains that were culturally, morphologically and antigenically typical of the species but were nonpathogenic for mice (De Jonckheere *et al*, 1974). These became known as "nonpathogenic *N. fowleri*" and prompted speculation as to whether these could become virulent in response to certain environmental factors (De Jonckheere & Van de Voorde, 1977b). Stevens *et al*, (1980) examined these strains and found them to be distinct from *N. fowleri* in their agglutination by the plant lectin concanavalin A and also their nuclear and cytoplasmic ultrastructure. Subsequent studies using isoenzyme and DNA analysis have confirmed the distinction of the species (De Jonckheere, 1982a; Daggett & Nerad, 1983; Kilvington *et al*, 1984; Pernin, 1984; De Jonckheere, 1987b & 1987c; Clark *et al*, 1989). *N. lovaniensis* is found in thermal aquatic environments, usually in association with *N. fowleri* (Stevens *et al*, 1980; De Jonckheere *et al*, 1983; De Jonckheere, 1987a; Kilvington *et al*, 1991b). The detection of the species in an environment is, therefore, an indicator of conditions favourable for the presence of *N. fowleri* (De Jonckheere, 1982b).

N. australiensis (De Jonckheere, 1981). In 1973, a strain of *Naegleria* was isolated from Australia that did not react with antiserum against *N. fowleri* but could grow at 42°C. Furthermore, the strain was pathogenic for laboratory mice but less virulent than *N. fowleri*. By these criteria, and from unique isoenzyme electrophoretic patterns, the species *N. australiensis* was proposed (De Jonckheere, 1981). The isolation of *N. australiensis* has been reported worldwide (Scaglia *et al*, 1983; Michel & De Jonckheere, 1983; Pernin & De Jonckheere, 1984; Derr-Haff & De Jonckheere, 1984; John & De Jonckheere, 1985). The species is commonly found in England in natural waters warmed by solar energy during the summer months, water cooling towers and also in tropical fish tanks, frequently in high numbers (S. Kilvington, unpublished observations). To date, no reported case of human infection has been attributed to *N. australiensis*.

N. australiensis italica (De Jonckheere *et al*, 1984a). Isolates of a highly virulent *Naegleria* were made from a therapeutic thermal spa in Northern Italy. These were initially identified as *N. fowleri*. On re-examination of 14 of these strains, De Jonckheere noted that they could tolerate growth only up to 42°C, reacted to titre with *N. australiensis* antiserum, did not agglutinate with concanavalin A (unlike *N. australiensis*) and were distinct in their isoenzyme electrophoretic profiles. The subspecies of *N. australiensis*, *N. australiensis italica* was therefore proposed in 1984 (De Jonckheere *et al*, 1984a). *N. australiensis italica* has only been isolated from the spa source in Italy and no instances of human infection have been described.

N. andersoni and *N. andersoni jamiesoni* (De Jonckheere, 1988a). De Jonckheere in 1988 reported *Naegleria* strains that tolerated growth up to 40°C, were nonpathogenic and showed unique isoenzyme profiles and whole-cell DNA restriction fragment length polymorphisms (RFLPs). The strains were isolated from environmental samples from Australia and also from water containing tropical fish imported into Belgium from South East Asia. Because of the apparent global distribution of these strains, the species *N. andersoni* with two subspecies *N. andersoni andersoni* and *N. andersoni jamiesoni* was proposed (De Jonckheere, 1988a).

The key distinguishing biological characteristics which currently define the genus *Naegleria* are given in Table I.

Table I. Key differentiating characteristics of the *Naegleria* species

Characteristic		*N. fowleri*	*N. lovaniensis*	*N. australiensis*	*N. aust. italica*	*N. andersoni*	*N. and. jamiesoni*	*N. gruberi*	*N. jadini*
Temp tolerance on NNA-*E. coli*		45°C	45°C	42°C	42°C	40°C	40°C	≤37°C	<37°C
Tissue culture cytopathogenicity	32°C	+	+	+	+	+	+	+	+
	37°C	+	+	+	+	+	+	-	-
Pathogenicity	mice	+	-	+	+	-	-	-	-
	man	+	-	-	-	-	-	-	-
Agglutination by concanavalin A		-	+	-	+	?	?	?	?
Naegleria spp. antiserum	polyclonal	±	±	+	+	?	?	?	?
	monoclonal	+	?	?	?	?	?	?	?
Unique isoenzymes/DNA RFLPs		+	+	+	+	+	+	+	+

? = not known

± = significant cross reaction to *N. lovaniensis* with *N. fowleri* polyclonal sera and visa versa

1.9. Morphological characteristics of the *Naegleria*

The genus *Naegleria* is characterised by a life-cycle of trophozoite, cyst and temporary, non-feeding and non-dividing flagellate (Page, 1988). The trophozoites of *N. fowleri* are approximately 10 to 20 μm in length and move in a slug-like ("limax") manner by protrusion of distinct hyaline pseudopodia. The rate of movement increases with temperature and also electrolyte concentration (King *et al*, 1983). A trophozoite of *N. fowleri* is shown in Figure 1.

Cysts are formed from the trophozoites and are round, double walled, and vary in size from approximately 7 to 18 μm. The inner and outer cyst wall are parallel and occasional pores, through which the trophozoite excysts, join the walls. Cyst formation is probably in response to adverse changes in the environment, such as depletion of food source. Under laboratory conditions, encystment occurs on NNA-*E. coli* plates when the bacterial source is consumed. Excystment occurs within a few hours if the cysts are inoculated on to fresh NNA-*E. coli* medium (John, 1993). Cysts of *N. fowleri* are shown in Figure 2.

The flagellate stage is formed from the trophozoite and appears pear shaped with usually two flagella of equal length which produce a cork-screw pattern of swimming. Flagellate formation *in vitro* is induced by incubating trophozoites in a weak buffer or distilled water and can occur in as little as one hour (Fulton, 1970 & 1977). Flagellates neither feed nor divide and the process is reversible with conversion back to the trophozoite form. It has been suggested that in the natural environment the process occurs from the dilution of the aquatic habitat, perhaps by rain fall, and is a mechanism by which the organism can colonise fresh sites (Griffin, 1983; Warhurst, 1985). However, whilst the flagellates can swim at a speed of 100 μm per second, which would be equivalent to 9 meters per day, it is more likely that the organism is carried from one site to another by the action of flood water (Fulton, 1977 & 1993). A flagellate of *N. fowleri* is shown in Figure 3.

Figure 1 A trophozoite of *N. fowleri*

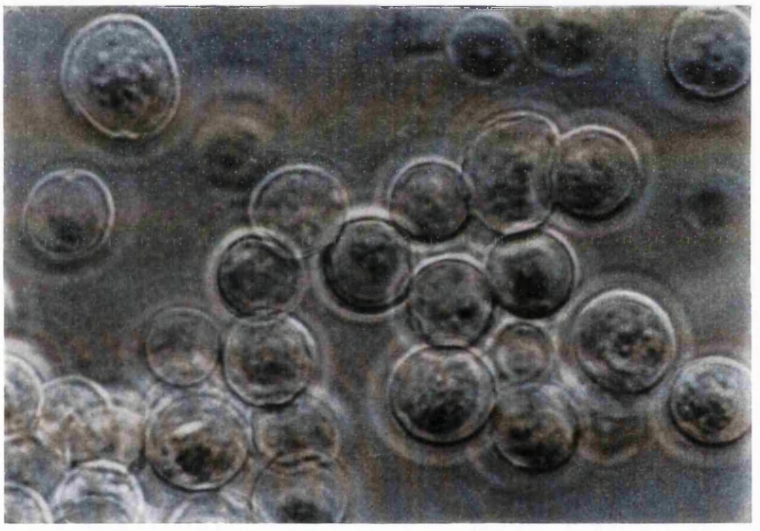

Figure 2 Cysts of *N. fowleri*

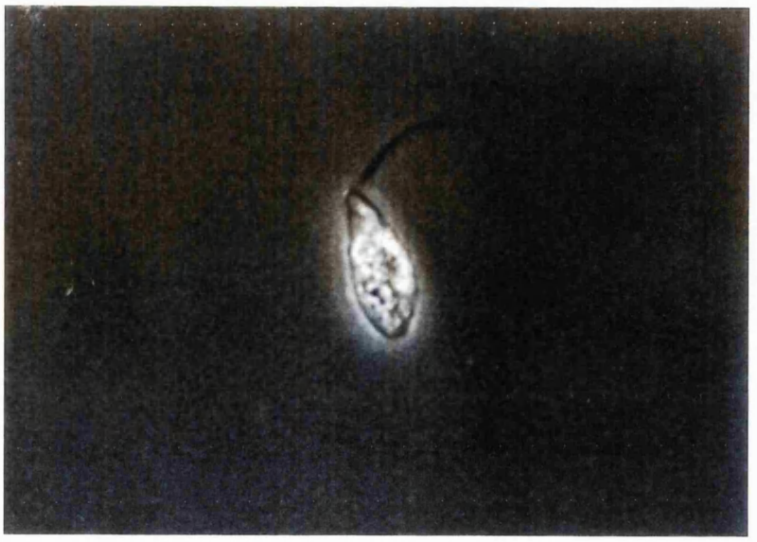

Figure 3 A flagellate of *N. fowleri*

1.10. The molecular biology of *Naegleria*

All free-living and parasitic protozoa have a genome consisting of chromosomal DNA contained within the nucleus of the cell, together with extrachromosomal mitochondrial or kinetoplast DNA in aerobic species (Gutteridge & Coombs, 1977). Mitochondria or kinetoplasts are not present in anaerobic protozoa such as *Entamoeba*, *Giardia*, *Phreatamoeba balamuthi* and *Trichomonas* (Gutteridge & Coombs, 1977; Chavez *et al*, 1986; Albach, 1989). The genome of *Naegleria* is composed of chromosomal, mitochondrial and rRNA plasmid DNA.

1.10.1. Chromosomal DNA

The nuclear DNA content of *N. gruberi* (NEG) has been estimated as 0.17 pg /cell, which equates to approximately 30 times that found with *E. coli* or 1/30th that of a mammal (Fulton, 1970; Clarke, 1990). Early estimates of the chromosome number in *Naegleria*, based on light microscopy, ranged from 3-16. However, with the advent of electron microscopy, it became apparent that the chromosomes were too small and closely packed to be accurately enumerated. With the development of pulse-field gel electrophoresis which enables the separation of megabase pairs (mbp) fragments of DNA, the chromosome number in *Naegleria* has been determined. De Jonckheere (1989) found the number of chromosomes to vary between 15 and 23 chromosomes for *Naegleria* spp, with differences occurring between strains of the same species. In another study, about 23 chromosomes ranging from 400 - >2,000 kilobase pairs (kbp) were found in two strains of *N. gruberi* (Clark *et al*, 1990). The position of genes on the chromosomes and their function is largely unknown. However, mapping of the actin genes showed them to be dispersed over several chromosomes, whilst the α-tubulin genes are almost all on one single chromosome (Clark *et al*, 1990).

An anomaly in these findings is that whilst the total chromosome size for *N. gruberi* (NEG) is approximately 19 mbp, this is no where near the expected chromosomal genome size of approximately 104 Mbp (Fulton, 1970). The reasons for such a difference are, at present, unclear. Clark (1990) suggests that the *Naegleria* may be polyploidy, although isoenzyme studies usually imply diploidy (Cariou & Pernin, 1987). Alternatively, individual chromosomes may be present at variable ploidy, as appears to be the case in *Giardia* (Adam, 1991).

1.10.2. Mitochondrial DNA (mtDNA)

Naegleria possess numerous mitochondria with a respiratory process similar to that found in mammalian cells (Weik & John, 1979; John, 1993). The mitochondrial genome of *N. gruberi* has been reported to comprise approximately 14% of the total DNA in the cell, and have a G+C content of 23% Fulton (1970). The mtDNA from several *Naegleria* species and one strain of *Vahlkampfia* has recently been isolated from purified organelles (Milligan & Band, 1988). By summation of restriction fragment lengths, the average *Naegleria* mtDNA size was estimated as 54.7 $\pm$ 2.9 kbp in length and there are about

420 copies per cell. Based on electrophoretic mobility, it was also proposed that the mtDNA is circular, as in the majority of other eukaryotes.

1.10.3. Ribosomal RNA (rRNA) genes

Clark and Cross (1987) first reported that the rRNA genes of *Naegleria* are carried on a 14 kbp plasmid. The extrachromosomal elements were shown to encode a single transcription unit for the small subunit 5.8S and large subunit rRNA. The 5S rRNA gene was, however, absent. Approximately 4,000 copies of the plasmid are present in each cell and account for some 17% of the total cell DNA. No evidence of a chromosomally integrated copy was found, indicating that the rDNA circles were most probably self-replicating.

Subsequent studies have shown that all Schizopyrenid amoebae (Page, 1988) contain rRNA plasmids (Clark & Cross, 1988a). It is unusual for eukaryotic cells to contain extrachromosomal DNA other that mtDNA (Rush & Misra, 1985). Prior to this observation in *Naegleria* and other Schizopyrenid amoeba, the presence of plasmid elements had only been reported in the slime mould, *Dictyostelium* (Metz *et al*, 1983; Orii *et al*, 1987) and the yeast, *Saccharomyces* (Broach, 1981). Linear extrachromosomal elements have also been reported in *Euglena* (Ravel-Chapuis *et al*, 1985) and *Physarum* (Voght & Braun, 1976). Most recently, circular plasmid elements have also been detected in *E. histolytica* (Bhattacharya *et al*, 1989; Huber *et al*, 1989).

The discovery of the rDNA of *Naegleria* and other Schizopyrenid amoebae has proved to be a useful region for the study of eukaryotic evolution and taxonomic status of the genus. Comparison of the rDNA nucleic acid sequence of *N. gruberi* NEG-M with that of other organisms showed that this species represented a relatively early branch in eukaryotic evolution, diverging between *Physarum* and *Entamoeba* (Figure 4). All species which precede *Naegleria* in the branch are flagellates, at least in part of their life cycle, and this has led to the conclusion that early eukaryotes were probably flagellates (Clark, 1990).

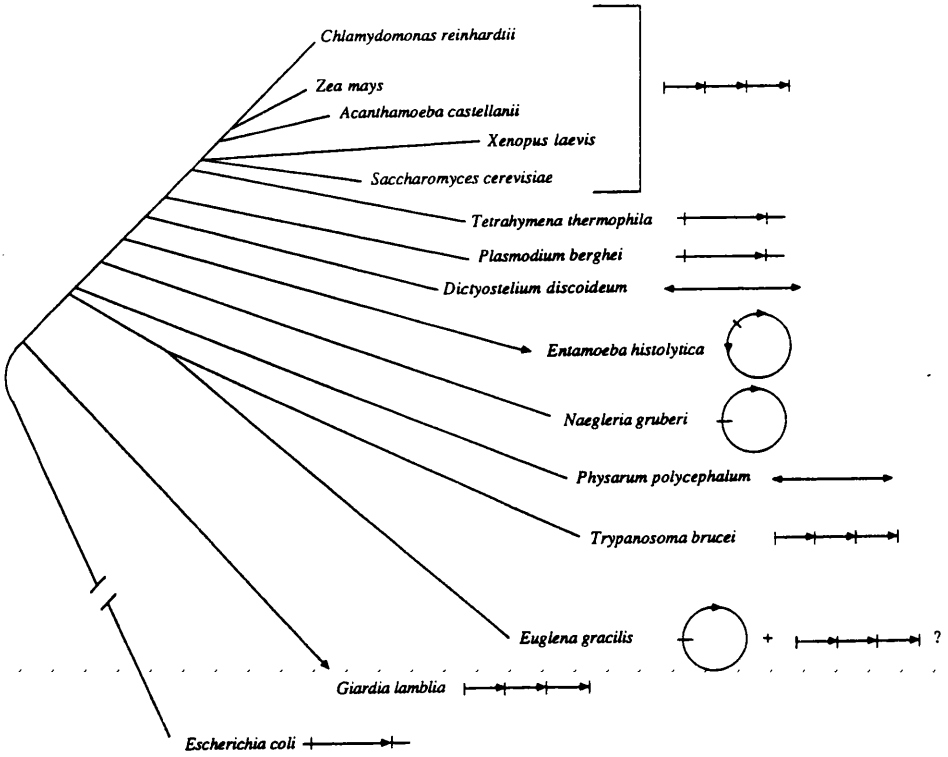

Figure 4 Evolutionary relationship of *N. gruberi* (NEG-M) to other eukaryotes based on small subunit rRNA sequence analysis (Clark, 1990)

As has been mentioned, all species of *Naegleria* and other vahlkamapfiid amoebae contain extrachromosomal rDNA plasmids which is present in high copy number within the cell. If total DNA extracted from these organisms is digested with restriction endonucleases (enzymes which cut DNA at site specific nucleotide sequences) and then subjected to agarose gel electrophoresis, a series of discrete bands of DNA are resolved termed restriction fragment length polymorphisms or RFLPs. These represent digestion products from both the rDNA and mtDNA. Such is the specificity of the RFLPs obtained, that genera, species and also strains can be differentiated (De Jonckheere, 1986; De Jonckheere, 1987c; De Jonckheere, 1988b; McLaughlin, *et al*, 1988; Milligan & Band, 1988). The most detailed study being that of Clark and colleagues (1989) who examined the relationship of all *Naegleria* species and related genera using a combination of RFLPs detected on agarose gel electrophoresis together with those obtained by hybridisation with cloned regions of the rDNA from *N. fowleri* and *N. gruberi*. The inter-species relationships were largely in agreement with those obtained by isoenzyme analysis (see *1.8. Taxonomy of the Naegleria*). In addition, the heterogenisity of strains assigned to the species *N. gruberi* was further confirmed with five distinct groups being identified (Figure 5).

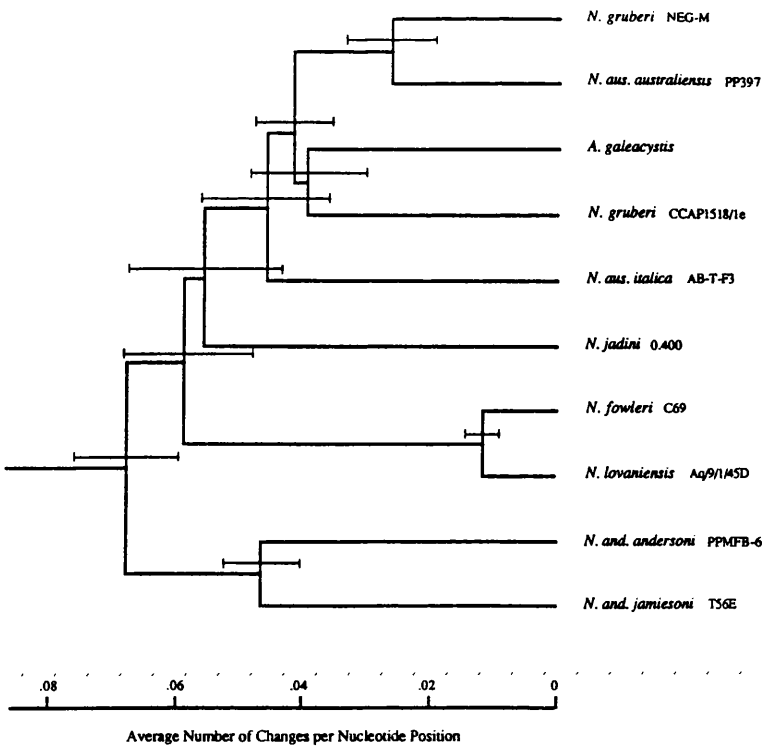

Figure 5 Phylogenetic tree of *Naegleria* spp. type strains based on DNA RFLPs (Clark *et al*, 1989)

1.10.4. A Group I intron in certain *Naegleria* species

Recently, it has been found that some species of *Naegleria* contain a Group I intron in the small subunit ribosomal RNA genes. This was first reported by Embley and colleagues (1992) in *N. andersoni andersoni* strain PPMFB-6. In this instance, amplification of the entire small subunit (SSU) ribosomal RNA gene from *Naegleria* species demonstrated a single band of approximately 2 kbp in *N. fowleri*, *N. lovaniensis*, and *N. australiensis* but in *N. australiensis italica*, *N. andersoni andersoni* and *N. andersoni jamiesoni* a band of approximately 3.2 kbp was found. Sequence analysis of the insertion showed it to contain motifs which identified a Group I intron. The presence of Group I introns in nuclear SSU rRNA genes are rare and have previously only been reported in *Ustilago maydis*, *Pneumocystis carini* and *Ankistrodesmus stipitatus* (cited in Embley *et al*, 1992). In a separate study De Jonckheere (1993) confirmed the presence of the Group I intron in these *Naegleria* species and also in some strains of *N. gruberi*.

1.10.5. Flagellate formation

No overview of the current understanding of *Naegleria* molecular biology would be complete without consideration of the dramatic changes that the trophozoite undergoes in the process of flagellate formation. This field of research has been dominated by Dr Chandler Fulton whose study of the subject extends over two decades. The fascination has been the remarkable features of the process: transformation occurs

rapidly, in about one hour; the process is reversible with conversion back to the trophozoite form; trophozoites which have been grown for years without transforming promptly do so when a stimulus is provided; such is the dramatic change in phenotype that the two forms could be mistaken for belonging to different classes of organism (Fulton, 1970, 1977 & 1993).

The flagella, composed of tubulin consisting of α and ß subunits, are assembled into the classical "9 + 2" arrangement for the axonemes. *Naegleria* trophozoites do not possess the components of the flagellar apparatus and must reorder themselves both biochemically and morphologically during transformation which involves going from an actin based trophozoite to a tubulin based flagellate. Although the amoebae use tubulin for the mitotic spindle, that which forms the flagellar microtubules is virtually all synthesised *de novo*. This process requires the synthesis of new RNA and protein and the switching on of new genes (Fulton, 1993).

It has been speculated that *Naegleria* flagellates are gametes for sexual reproduction, although supporting evidence is lacking (Fulton, 1993). However, Cariou and Pernin (1987) have concluded that isoenzyme patterns seen in *Naegleria* spp. isolates can only be explained if these organisms undergo genetic recombination. Further studies in this area to investigate the possibility of a sexual cycle in *Naegleria* are therefore warranted.

1.11. Primary amoebic meningoencephalitis in Bath Spa

In July 1978 an 11 year old girl was admitted to the Royal United Hospital, Bath with a three day history of headache, and a 1 day history of pyrexia, vomiting and blurred vision. A lumbar puncture showed turbid CSF which, under microscopic examination, indicated a pyogenic meningitis although no organisms were seen. Treatment was commenced with penicillin, chloramphenicol and sulphadimide to no effect and her condition worsened with seizures and respiratory arrest. Thirty-six hours after admission the initial CSF showed no growth and a second lumbar puncture was performed. This time microscopic examination of the CSF showed numerous motile amoebae and provided a provisional diagnosis of PAM to be made by Dr Paul Mann, Director of the laboratory. Despite the immediate change of antimicrobial therapy to amphotericin B the patient died 5 days after admission following a cardiac arrest (Cain *et al*, 1981).

Inoculation of the CSF into mammalian tissue culture cell lines and on to NNA-*E. coli* enabled the culture of the organism. This was identified as *N. fowleri* by Dr David Warhurst at the Hospital for Tropical Diseases, London on the basis of trophozoite and cyst morphology and flagellate formation. Subsequent studies using isoenzyme electrophoresis and DNA RFLP typing have confirmed the identification of the strain as *N. fowleri* (Kilvington *et al*, 1984; Kilvington & Beeching, 1989).

As with any case of PAM, the priority was to identify the source of infection. Here, it was realised that 6 days prior to her admission to hospital the patient had participated in a school swimming gala at the City's Beau Street baths (Figure 6). The swimming bath itself was fed with thermal water taken from the natural hot springs that rise in the City at the Roman Baths complex (Figure 7).

After consultation between Dr Paul Mann, Dr David Warhurst and the local Environmental Health Department, the immediate closure of the Beau Street and adjoining Old Royal swimming baths was ordered. The experience from Usti, Czechoslovakia where 16 fatal cases of PAM occurred over a 4 year period at a swimming pool that was allowed to remain open, demonstrated only too clearly what can happen if prompt action is not taken in such circumstances (Cerva & Novak, 1968; Kadlec *et al*, 1978).

It therefore became imperative to examine the source of the thermal springs beneath the King's Bath (Figure 8). This necessitated draining the bath and providing lighting and ventilation for the enclosed space where the springs rise. This revealed to the City Engineers that the foundations of the Pump Room were being severely eroded. Substantial underpinning of the buildings and measures to avoid further erosion were seen to be necessary. To facilitate this work it was decided to cut away the concrete floor of the King's Bath placed at the end of the 19th century (Figure 9). This in turn provided an opportunity to excavate the Roman Temple remains known to be lying beneath the Pump Room. As a consequence, important archaeological information about the springs' use in Roman times was uncovered together with many priceless artifacts from the period (Kellaway, 1991).

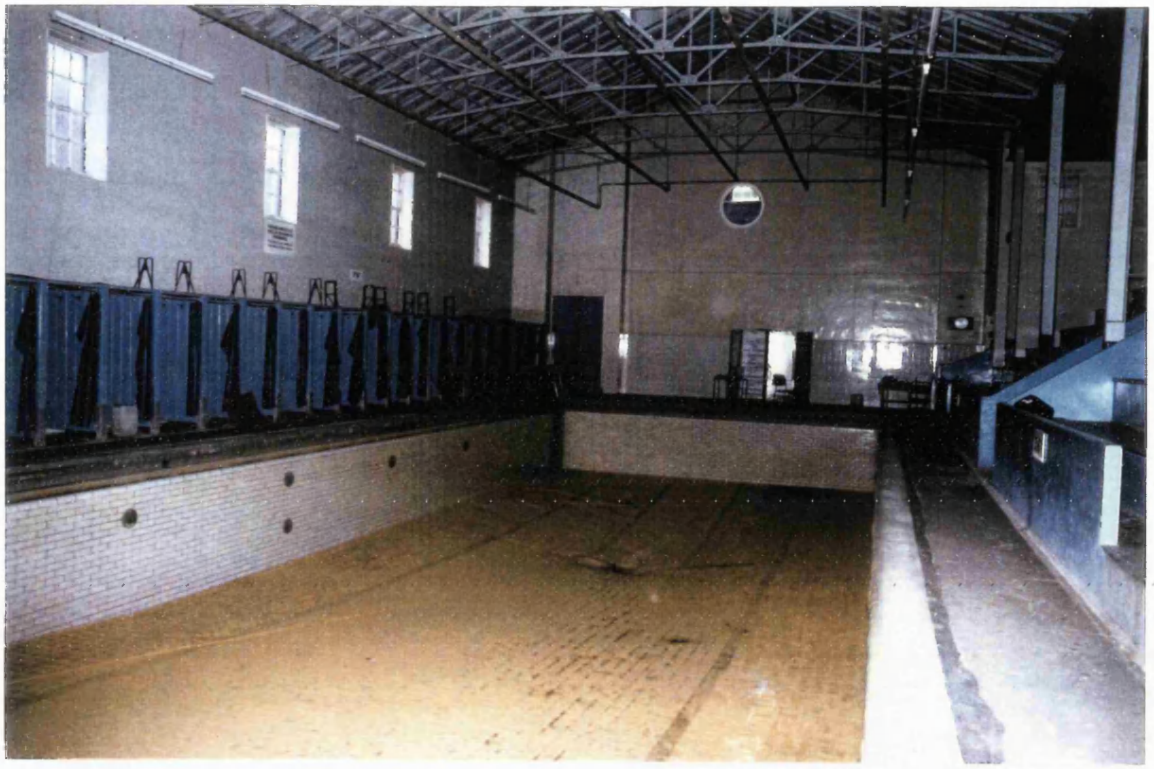

Figure 6 The Beau Street swimming bath in 1978

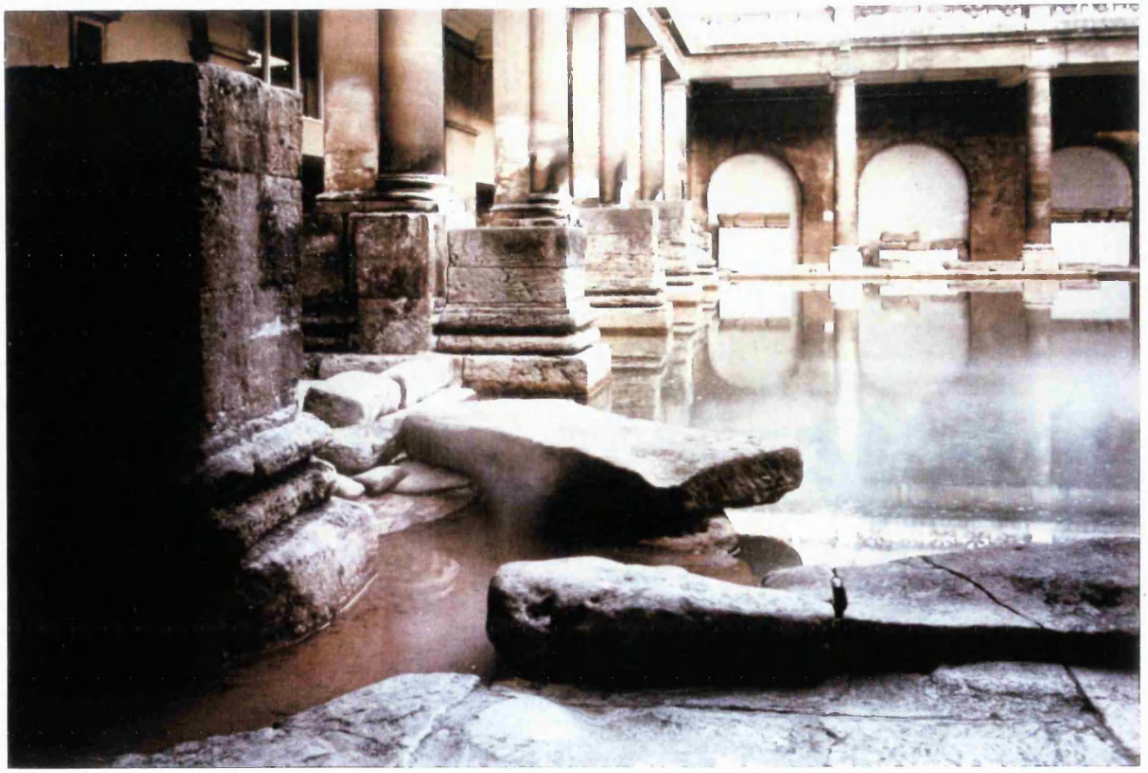

Figure 7 The Great Bath of the thermal springs complex

Figure 8 The King's Bath in 1978

Figure 9 The King's Bath drained and with part of the floor removed

Such excavations provided ample sample material and numerous isolates of thermophilic *Naegleria* were made which were morphologically, culturally and antigenically similar to *N. fowleri*. However, when the strains were later inoculated intranasally into mice (the definitive test at the time for the identification of *N. fowleri*) they were found to be nonpathogenic. This apparent presence of nonpathogenic *N. fowleri* was in common with findings from other workers around the world and led to the speculation that avirulent variants of the species occurred and that some environmental factor possibly triggered pathogenicity in the strains (De Jonckheere & Van de Voorde, 1977b). However, Stevens and colleagues (1980) in the USA showed that these nonpathogenic strains were biochemically distinct from *N. fowleri* and represented a new species *N. lovaniensis* (see *1.8. Taxonomy of the Naegleria*).

In the light of these findings, a fresh search for *N. fowleri* in the Roman Baths was initiated in the summer of 1981. In December of the same year mouse pathogenic strains of the *N. fowleri* were isolated from the north east corner of the Great Bath (Figure 10: point E), finally confirming that the thermal springs were the source of contamination in the fatal case of PAM at the swimming pool. Further systematic attempts to isolate *N. fowleri* from the Roman Baths complex continued but without success and it was not until June of 1983 that further isolates were made, this time from the point where the overflow water from the Great Bath and King's Bath meet (Figure 10: point F) before eventually passing into the River Avon (Kilvington *et al*, 1991b).

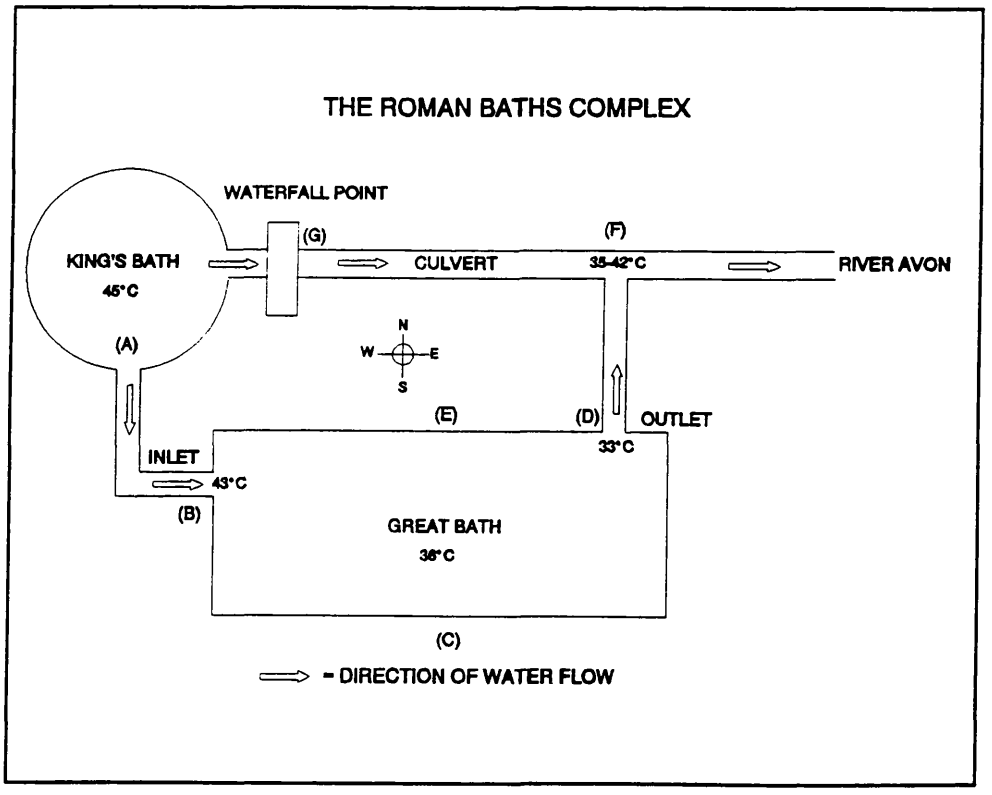

Figure 10 Diagram of the Roman Baths complex

These surveys indicated that:

i. The presence of *N. fowleri* around the thermal springs complex was extremely rare.

ii. *N. lovaniensis* and *N. australiensis* were the predominant thermophilic *Naegleria* in the hot springs and that for every isolate of *N. fowleri*, many hundreds of strains of the latter species had to be eliminated.

iii. Improved methods for the differentiation of these closely related species were desperately required.

As a result, several techniques were developed to facilitate the provisional differentiation of *N. fowleri* and *N. lovaniensis* soon after primary isolation. These included: cellulose acetate membrane electrophoresis with respect to aspartate aminotransferase (Kilvington *et al*, 1984); specific differences in intracellular enzyme activities (Kilvington & White, 1985); antigenic detection (Kilvington & White, 1986); and the use of a semi-selective culture medium (Aufy *et al*, 1986).

Although these new techniques served a useful purpose at the time, they did not lend themselves to the routine examination of the large numbers of isolates frequently encountered in samples taken from the hot springs, or else were not specific enough to allow the unequivocal identification of *N. fowleri*. Recent advances in molecular biology have greatly simplified the procedures by which organism DNA can be isolated, cloned into bacteriophages or bacterial plasmids and analyzed (Sambrook *et al*, 1989). The cloned DNA can be labelled by the incorporation of radioactive nucleotides such as 5'-[α-^{32}P] deoxycytidine triphosphate or by novel non-radioactive methods and used as a probe to detect complementary DNA by hybridisation. Such nucleic acid probes can be a highly specific and rapid means of identifying pathogenic microbes (Coates, 1986). More recently, a major progress in the diagnostic use of DNA probes has been through the development of the polymerase chain reaction (PCR). The PCR is an *in vitro* amplification procedure that can, in a matter of hours, isolate and amplify a specific segment of DNA by as much as 10^8-fold (reviewed by Oste, 1988). PCR amplification involves two synthetic oligonucleotide primers that flank the DNA segment to be amplified. These primers are designed to hybridise to opposite strands of the target sequence and DNA synthesis (using the enzyme DNA Taq polymerase) occurs across the region of the primers. Successive cycles of DNA synthesis results in a vast amplification of DNA that can be readily detected by direct visualisation after gel electrophoresis or in combination with probe hybridisation. Thus target DNA (ie. from a specific pathogen) can be rapidly amplified to detectable levels within a matter of hours.

1.12. Aims of the study

The opportunity was seen to apply these molecular biological techniques to the development of rapid and specific methods for the detection of *N. fowleri* from the environment. To this end, the following research objectives were undertaken:

i. Clone regions of *N. fowleri* DNA for use as probes in the sensitive and specific detection of the organism.

ii. Sequence such probes in the development of a PCR test for the identification of *N. fowleri*.

iii. Evaluate the potential for the DNA probes and PCR for the rapid and specific identification of *N. fowleri* from the environment.

2. THE CULTURE AND CRYOPRESERVATION OF *NAEGLERIA*

2.1. Introduction

In the natural environment FLA feed on a variety of bacteria, fungi, algae and other protozoa. Early attempts to isolate FLA from the environment used nutrient agar which stimulated the growth of associated bacteria and so provided a food source for the amoebae (Cutler *et al*, 1922; reviewed in Singh, 1975). The problem encountered with this technique was that it encouraged the growth of bacteria that were either inhibitory to FLA or else favoured as a food source by only certain species. Singh first developed the use of non-nutrient agar (NNA) seeded with a living suspension of a single bacterial species for the isolation of FLA (cited in Singh, 1975). After examining many species of bacteria an *Aerobacter* sp. (strain 1912) was found to be most suitable for the growth of a variety of FLA (cited in Singh, 1975). This method of monoxenic culture has since formed the basis of all methods for the isolation and maintenance of FLA with *A. aerogenes* or *E. coli* being most widely used bacterial food source (Page, 1988; Cerva, 1980a). Typically, NNA at a concentration of 1.5% in distilled water or Page's amoeba saline is used (Page, 1988). However, ¼ strength Ringer's solution is also suitable (S. Kilvington, personal observation).

Although monoxenic culture on NNA readily allows the primary isolation and maintenance of FLA, the cell yields are low and the trophozoites encyst freely. Biochemical and molecular studies usually require large numbers of trophozoites grown under controlled conditions. For this purpose the amoebae have to be adapted to axenic (bacteria-free) growth in liquid media. Cailleau (1933) first grew a free-living amoeba axenically by culturing *A. castellanii* in a simple peptone-carbohydrate broth medium. The first axenic culture of a *Naegleria* was obtained by Balamuth (1964) with *N. gruberi* and by Cerva (1969) for *N. fowleri*. Since this time several media have been described for the axenic culture of *Acanthamoeba* and *Naegleria* (Neff, 1957; Nelson & Jones, 1970; Culbertson, 1971; De Jonckheere, 1977; Haight & John, 1980; Laverde & Brent, 1980; Stevens *et al*, 1981). Defined chemical media for the growth of *Acanthamoeba* (Byers *et al*, 1980) and *Naegleria* (Nerad *et al*, 1983) have also been reported.

Cerva (1980b) reported the axenic culture of *N. fowleri* in a simple medium consisting of 2% casitone supplemented with 10% horse serum. However, media for the growth of *Acanthamoeba* and other *Naegleria* spp. are generally complex and usually comprise a peptone source, yeast extract, glucose and serum (either horse, bovine calf or foetal calf). For *N. gruberi*, it has been shown that haemin can be used in place of a serum source (Band and Balamuth, 1974). Serum is not generally required for the growth of *Acanthamoeba*, although its presence enhances the rate of cell growth and yield (Stevens & O'Dell,1973). Chang (1974) showed that luxuriant axenic growth of *N. fowleri* occurred in a medium consisting of serum, casein, glucose, yeast extract and foetal calf serum (SCGYM). This medium has subsequently been shown to support the growth of all *Naegleria* spp., many *Acanthamoeba* spp. and several other FLA (De Jonckheere, 1983; De Jonckheere, 1986; De Jonckheere, 1987c). In this medium,

it has been observed that *N. lovaniensis* grows less well than *N. fowleri* at 37°C or not at all at 43°C and has been proposed as a provisional means of differentiating the species (De Jonckheere, 1977; Aufy *et al*, 1986).

At the Bath Public Health Laboratory it has been found that SCGYM supplemented with 0.1% filter sterilised Panmede liver digest (#SCGYM) gives improved growth of all *Naegleria* spp., *Willaertia magna* and many *Acanthamoeba* spp. However, Panmede liver digest is no longer available commercially and has prompted the examination of alternative media for the growth of these organisms. To this end, it was found that the yeast extract-peptone-yeast nucleic acid-folic acid-haemin (YPNFH) medium described by Laverde and Brent (1980) for the culture of *N. gruberi*, when modified by the addition of 0.1% glucose and 10% heat inactivated (56°C for 30 minutes) foetal calf serum (#YPNFH), was as good as #SCGYM in this respect.

Whilst the trophozoites of FLA can be easily grown on NNA-*E. coli* or one of the several semi-defined broth media listed above, it is impractical to maintain numerous strains by continuous culture. Besides the expensive and labour intensive nature of the process, cultures can become mislabelled or contaminated through the regular subculturing procedures. Continuous culture, particularly of axenic strains, can also result in the loss of biological characteristics such as virulence and ability to encyst or flagellate (Stevens & O'Dell, 1974; Wong *et al*, 1977; Marciano-Cabral, 1988). This prompted the investigation of methods for the long-term cryopreservation of both pathogenic and nonpathogenic FLA. Based on a method described previously for the cryopreservation of the anaerobic parasite *Trichomonas vaginalis* (Miyata, 1975), it was found that the trophozoites of *Naegleria*, *Acanthamoeba* and other FLA could be successfully cryopreserved from axenic or bacterial cultures using a two-step cooling process in standard laboratory freezers (Kilvington & White, 1991). Dimethylsulphoxide (DMSO) at a final concentration of 5% is used as the cryoprotectant and cooling achieved by placing the ampoules directly at -20°C for 60 minutes, followed by a further 60 minutes at -70°C. The ampoules are then either held at -70°C or transferred to liquid nitrogen. Recovery is achieved by rapid thawing at 37°C and inoculation into fresh culture media. Although relatively low recovery rates are obtained, suggesting that conditions are not optimal, the method has been found to be a simple and reproducible means of cryopreserving pathogenic and nonpathogenic FLA.

The ability to culture and store a wide variety of FLA is fundamental to the overall objectives of this project to develop molecular techniques for the identification of *N. fowleri*. Therefore, the methods used for culturing FLA both under monoxenic and axenic conditions, and the procedures for cryopreserving these organisms are given here in detail.

2.2. Materials and Methods

2.2.1. The culture of FLA

2.2.2. Monoxenic culture on NNA-*E. coli*

NNA seeded with *E. coli* (NNA-*E. coli*) was prepared as described in **Appendix 1.1**. *E. coli* strain JM101 was used as the bacterial food source. Trophozoites of all FLA were grown in air at 32°C and subcultured every 4-5 days. Regions of encysted amoebae on the plates can be cut out and stored in screw-capped bottles at 4°C for several months (if not longer). However, growth of contaminating moulds and yeasts can be a problem.

2.2.2.1. Axenic culture

To adapt strains to axenic growth, trophozoites were washed from the an NNA-*E. coli* plate with ¼ strength Ringer's solution and pelleted by centrifugation at 500 x g for 5 minutes at room temperature. The cell pellet was then inoculated into flat sided tissue culture tubes (Nunc, Gibco, Middlesex, England) containing 3 ml of #SCGYM or #YPNFH (**Appendix 1.2 - 1.3**) and incubated in air at 32°C. After 48 hours the medium was gently poured from the tubes and replenished. Once axenic growth was established, as judged by monolayer formation in the tubes, the cultures were passaged through progressively larger tissue culture flasks (25 cm^2, 75 cm^2 and 180 cm^2) to provide sufficient cells for analysis. Typical trophozoite cell densities in #SCGYM or #YPNFH for *N. fowleri* were approximately 1-1.5 x 10^6 /ml and 6-8 x 10^5 /ml for other species. *N. fowleri* and *N. lovaniensis* usually adapt readily to axenic culture although several attempts may be necessary for other species.

2.2.3. Cryopreservation of *Naegleria* and other FLA

2.2.3.1. Requirements:

1. NNA-*E. coli* and #SCGYM or #YPNFH.

2. DMSO cryopreservation solution. To 9 ml of #SCGYM or #YPNFH add 1 ml of DMSO (10% final concentration) in a polypropylene tube (DMSO dissolves polystyrene). Mix thoroughly and store at 4°C in the dark for use within one week.

3. ¼ strength Ringer's solution (Oxoid, Basingstoke, England). Dissolve one tablet of Ringer's salts in 500 ml of dH_2O, dispense in 100 ml volumes and autoclave at 121°C for 15 minutes. Store at room temperature.

4. Two freezers set at -20°C and -70°C.

5. Liquid nitrogen storage container, ampoules and aluminium canes.

2.2.3.2. Axenic trophozoites

1. A tissue culture tube containing a semi-confluent growth of trophozoites is chilled on ice for 10 minutes and the amoebae harvested by centrifugation at 500 x g for 5 minutes at room temperature.

2. Resuspend the cell pellet 2 ml of #SCGYM or #YPNFH at room temperature and aliquot in 0.5 ml volumes into 1.2 ml screw capped, polypropylene ampoules measuring 41 mm x 12 mm (Nunc, Gibco, Middlesex, England).

3. Add 0.5 ml of the DMSO cryopreservation solution, equilibrated to room temperature, to each ampoule and mix by inversion three times.

4. Immediately place the ampoules upright into the bottom of a -20°C freezer and leave for 60 minutes (see *note 4*).

5. Transfer the ampoules to a -70°C freezer and leave for a further 60 minutes (see *note 4*).

6. Either store the ampoules at -70°C or attach to aluminium canes and plunge into liquid nitrogen.

7. To recover strains, remove an ampoule from the liquid nitrogen and place into an enclosed container under an operating microbiological safety cabinet and leave for 3 minutes (see *note 1*). This procedure is not necessary for strains stored at -70°C.

8. Place the ampoule in a 37°C water bath and invert occasionally to hasten thawing.

9. When thawed, inoculate the ampoule contents into 3 ml of prewarmed #SCGYM or #YPNFH and incubate at 32°C (see *note 2*).

10. After 2 hours, gently pour off the liquid from the culture tubes and replace with 3 ml of fresh, prewarmed medium and reincubate.

2.2.3.3. Monoxenic trophozoites

1. Flood the NNA-*E. coli* plate with 5 ml of ¼ strength Ringer's solution and gently suspend the trophozoites by rubbing the agar surface with a bent glass rod or cotton tipped swab.

2. Pipette the trophozoites into a screw capped tube and centrifuge at 500 x g for 5 minutes at room temperature.

3. Resuspend the cell pellet 1 ml of #SCGYM or #YPNFH at room temperature and aliquot in 0.5 ml volumes into 1.2 ml screw capped, polypropylene ampoules.

4. Follow steps 3-6 above (**2.2.3.4. Axenic trophozoites**).

5. To recover the trophozoites follow steps 7 and 8 above (**2.2.3.5. Axenic trophozoites**).

6. When thawed, inoculated approximately 0.5 ml on to the surface of a NNA-*E. coli* plate. Allow the liquid to absorb and incubate (see *note 2*).

2.2.3.6. Cysts

1. Flood the NNA-*E. coli* plate with 5 ml of ¼ strength Ringer's solution and suspend cysts by rubbing the agar surface with a bent glass rod or cotton tipped swab (some effort may be required to dislodge the cysts).

2. Pipette the cysts into a screw capped tube and centrifuge at 500 x g for 5 minutes at room temperature.

3. Resuspend the cell pellet in 1 ml of ¼ strength Ringer's solution and aliquot in 0.5 ml volumes into 1.2 ml screw capped, polypropylene ampoules.

4. Add 0.5 ml of the DMSO cryopreservation solution, equilibrated to room temperature, to each ampoule and mix by inversion three times.

5. Place upright into a -20°C freezer for storage at this temperature.

6. To recover the cysts, thaw an ampoule in a 37°C circulating water and spread approximately 0.5 ml on to the surface of a NNA-*E. coli* plate. Allow the liquid to absorb and incubate (See *note 2*).

2.2.4. Cryopreservation using the Nalgene™ Cryo 1°C Freezing Container (BDH, Leicester, England)

1. Remove high-density polyethylene ampoule holder and foam insert from the unit.

2. Add isopropanol (~250 ml) to the fill line (do not overfill).

3. Replace foam insert and ampoule holder.

4. Prepare trophozoites for cryopreservation from axenic or monoxenic cultures as described above for the two-step cooling process (i.e. resuspend in #SCGYM or #YPNFH with DMSO at a final concentration of 5%).

5. Place ampoules into the holder.

6. Place unit in bottom of a -70°C freezer and leave undisturbed for at least 4 hours or overnight.

7. Remove ampoules and plunge into liquid nitrogen for long term storage.

2.3. Notes

1. *N. fowleri* and many *Acanthamoeba* are pathogenic to man. All manipulations of these organisms should be done under appropriate containment facilities using a microbiological safety cabinet (Anon, 1990). Liquid nitrogen can also penetrate the washer sealing between the ampoule cap and tube and produce an explosion on warming. Ampoules should therefore be placed inside an enclosed container for 3 minutes immediately after removal from liquid nitrogen.

2. *Naegleria, Acanthamoeba* and most other FLA grow well on NNA-*E. coli* medium and produce zones of dense trophozoite growth that radiate away from the site of inoculation on the culture plate. Cysts are formed during prolonged incubation (5-7 days) of the plates. NNA-*E.coli* culture plates are incubated in sealed polythene bags to prevent drying. The temperature of incubation depends upon the species of FLA. All pathogenic species will grow at 37°C or above and most others at 32°C (Page, 1988).

3. A tissue culture tube containing an almost confluent growth of amoebae in #SCGYM or #YPNFH contains approximately 5 x 10^5 - 1 x 10^6 trophozoites /ml and is sufficient for preparing 4 ampoules for cryopreservation. The yield of trophozoites and cysts from NNA-*E. coli* medium are much lower and only 2 ampoules should be prepared per culture plate.

4. Freezers used for cryopreservation should not have been opened for at least 1 hour before use and should remain closed during the cooling steps. Ampoules should be transported between the freezers and liquid nitrogen tank inside a small enclosed polystyrene box to prevent warming.

5. For the Nalgene™ Cryo 1°C Freezing Container, trophozoites and cysts are prepared for cryopreservation using 5% DMSO, without prior equilibration, exactly as described above. The unit is stored at room temperature when not in use and the isopropanol is replace every fifth use.

2.4. Discussion

A study of the recovery rates of *Naegleria*, and *Acanthamoeba* trophozoites cryopreserved in liquid nitrogen by the two-step cooling method detailed here gave: *N. fowleri* 8 ±3%, *N. lovaniensis* 12 ±3%, *N. gruberi* 25 ±6% and *A. polyphaga* 34 ±5.5% from axenic cultures, and 21 ±7%, 31 ±10%, 44 ±11%, 49 ±9% respectively from monoxenic cultures (Kilvington & White, 1991). While these figures are low, particularly for axenic cultures of *N. fowleri* and *N. lovaniensis*, confluent growth of trophozoites is usually obtained within 2-3 days of incubation.

The method for the cryopreservation of FLA cysts at -20°C is based on a previously described method (Badenoch *et al*, 1990) for *Acanthamoeba* except that 5% DMSO is used instead of 1.5 mol/L methyl alcohol. Although not extensively tested, several *Naegleria* and *Acanthamoeba* strains cryopreserved by this method have been successfully recovered after 1-2 years storage.

The relatively low recovery rate following cryopreservation suggests that the methods are not optimal for FLA. The rate of cooling is an important factor in the cryopreservation of free-living and parasitic protozoa, with values of approximately 1°C min^{-1} to -60°C or -70°C seeming to be optimal (Warhurst & Wright, 1979; Farri *et al*, 1983; James, 1984; Leeson *et al*, 1984). This can be achieved using programmable cooling machines (Farri *et al*, 1983; James, 1984;) or suspending ampoules by a holding device in the vapour phase of liquid nitrogen of a dewar vessel (Warhurst & Wright, 1979; Kasten & Yip, 1976). Improved results may therefore be obtained if the cooling rate could be controlled at 1°C min^{-1} to -70°C using such methods.

Although DMSO at a concentration of 5-10% is the most widely used protectant for the cryopreservation of both free-living and parasitic protozoa, other agents such as glycerol and methanol have been used (James, 1984; Leeson *et al*, 1984). In addition, an equilibration period for the cells at different temperatures in the cryoprotectant is often employed (James, 1984). Studies addressing these variables are therefore suggested and may lead to improved viable recovery rates for FLA.

The Nalgene™ Cryo 1°C Freezing Container has only recently been obtained for the cryopreservation of FLA and viable recovery rates have not been defined. However, strains of axenically grown *N. fowleri* cryopreserved with the unit seem to be recovered with greater viability than the two-step cooling procedure, as judged by the numbers of motile trophozoites observed 24 hours after recovery from liquid nitrogen. From the manufacturer's data sheet, a cooling rate of approximately 1°C min^{-1} is obtained between 30°C and -50°C. This may be a more optimal cooling rate for the trophozoites of *N. fowleri*, at least, and so account for the improved recovery rates.

Besides *Naegleria* and *Acanthamoeba*, FLA of the genera *Hartmannella*, *Vahlkampfia*, *Willaertia* and *Vannella* have also been successfully cryopreserved by the two-step cooling method described here with viable recovery after 4 years storage in liquid nitrogen and 2 years at -70°C. This suggests the method may be suitable for the cryopreservation of many other genera of FLA. Although the Nalgene™ Cryo 1°C Freezing Container has not yet been extensively tested, initial findings suggest it may well provide a more optimal cryopreservation method. This feature, together with the convenience of the system, indicates that it may be the preferred method for the cryopreservation of the these organisms. Both techniques are simple and reproducible and use freezers that may be considered standard in most laboratories. Importantly, the problems associated with maintaining numerous strains by continuous culture are eliminated.

3. IDENTIFICATION OF *NAEGLERIA* SPECIES USING CELLULOSE ACETATE MEMBRANE ELECTROPHORESIS OF GLUCOSE PHOSPHATE ISOMERASE

3.1. Summary

Cellulose acetate membrane electrophoresis (CAME) with respect to glucose phosphate isomerase (GPI) was used to investigate the relationship between strains of *Naegleria* species and *Willaertia magna*. A single GPI band was shown by each species, the relative mobility of which allowed the differentiation between *N. fowleri*, *N. lovaniensis*, *N. australiensis* *N. andersoni andersoni*, *N. andersoni jamiesoni*, and *W. magna*. *N. australiensis* sub sp *italica* and *N. jadini* showed a similar GPI mobility. No interstrain variation in GPI profile was detected within each species, regardless of whether the strains were cultured with live or heat-killed *Escherichia coli* or in a semi-defined axenic broth medium. The technique is rapid, reproducible and simple to perform, and is proposed as a useful means of identifying *N. fowleri* soon after primary isolation from the environment.

3.2. Introduction

N. fowleri PAM is almost invariably fatal and is usually acquired from bathing in thermally polluted fresh water (John, 1982, Warhurst, 1985). It is therefore important to identify habitats containing the organism in the interests of preventive public health microbiology. This requires methods that are both accurate and reliable for the differentiation of the species from other closely related thermophilic *Naegleria*. As discussed in *1.8. Taxonomy of the Naegleria*, *N. lovaniensis* resembles *N. fowleri* in tolerating growth up to 45°C, cytopathogenicity for tissue culture cells and antigenic structure (Stevens *et al*, 1980). Mouse pathogenicity testing was originally used for identifying *N. fowleri*, where by intranasal or intracerebral inoculation with the organism produces 100% mortality in 3-7 days (Carter, 1970; John, 1982). However, this approach was found to be time consuming, expensive and did not lend itself to the examination of numerous environmental isolates. With the recognition of *N. australiensis* which was shown to be pathogenic for mice, albeit less so than *N. fowleri* (De Jonckheere, 1981), the test was rendered nonspecific.

Several techniques have been investigated for the differentiation of *Naegleria* spp. The plant lectin concanavalin A was shown to agglutinate trophozoites of *N. lovaniensis* but not those of *N. fowleri* or *N. australiensis* (Stevens *et al*, 1980; De Jonckheere, 1981). Polyclonal antibodies raised against *N. fowleri* will not recognise *N. australiensis* but do cross-react significantly with *N. lovaniensis* (De Jonckheere *et al*, 1974; Stevens *et al*, 1980; Kilvington & White, 1986). Monoclonal antibodies have been produced against *N. fowleri* and while they are highly specific, and offer (potentially) rapid means of detection (Visvesvara *et al*, 1987), they do not allow the differentiation of other members of the genus.

Characterisation of *Naegleria* whole-cell restriction fragment length polymorphisms (RFLPs), whilst highly specific for the differentiation of species, sub-species and strains (De Jonckheere, 1987c), is time consuming, technically demanding, requires specialised equipment and is not suited to the examination of large number of isolates which may be met during environmental surveys. The development of a polymerase chain reaction (PCR) test for *N. fowleri* has been reported (Mclaughlin *et al*, 1991; Sparagano, 1993b) but, as with monoclonal antibodies, it does not allow the simultaneous identification of other species.

The need for a reliable test that differentiated *Naegleria* spp. by specific biological characteristics was met by the application of isoenzyme electrophoresis. Here, cell lysates are electrophoresed in a supporting matrix that separates isoenzyme forms by differences in electrical charge and molecular size. The isoenzyme bands are then detected by incubation with an appropriated enzyme substrate linked with a chemical stain to visualise the reaction (Shaw & Prasad, 1970). Isoenzyme electrophoresis is a potent technique for the differentiation of both prokaryotic and eukaryotic organisms (Shaw & Prasad, 1970; Selander *et al*, 1986) including the parasitic protozoa *Entamoeba* (Reeves & Bishceoff, 1968; Sargeaunt & Williams, 1978a & 1978b; Sargeaunt *et al*, 1980; Mirelman *et al*, 1986), *Leishmania* (Gardiner *et al*, 1974), *Giardia* (Bertram *et al*, 1983; Baveja *et al*, 1986) and *Trichomonas vaginalis* (Soliman *et al*, 1982).

Warhurst and Thomas (1978) first used isoenzyme electrophoresis in the study of *Naegleria*, revealing isoenzyme differences with respect to glucose phosphate isomerase in two strains of *N. gruberi*. Subsequent application of the technique was used to differentiate strains of *N. fowleri* and *N. gruberi* (Nerad & Daggett, 1979; Visvesvara & Healy, 1980), *N. fowleri* and *N. lovaniensis* (De Jonckheere, 1982a; Kilvington *et al*, 1984), and for the recognition of new species (De Jonckheere, 1981; De Jonckheere *et al*, 1984a & 1984b; De Jonckheere, 1988a). Several studies have confirmed the value of isoenzyme electrophoresis in the identification and phylogenetic relationship of *Naegleria* spp. (Daggett & Nerad, 1983; Pernin *et al*, 1985; Moss *et al*, 1988; Pernin and Cariou, 1989).

A variety of separating gels have been used for the isoenzyme analysis of *Naegleria*: starch (Nerad & Daggett, 1979; Daggett & Nerad, 1983), polyacrylamide (Visvesvara & Healy, 1980; Moss *et al*, 1988), agarose (De Jonckheere, 1982a; Pernin, 1984) or cellulose acetate (Robinson *et al*, 1992). While these methods have proved highly effective in this respect, the gels can be difficult to prepare and require lengthy electrophoresis times and large volumes of expensive detection reagents. In addition, strains usually require adaptation to axenic culture before testing, which can be a difficult and protracted process. Moss and Mathews (1987) described the use of cellulose acetate membrane electrophoresis (CAME) for the rapid and simple isoenzyme differentiation of invasive and non-invasive strains of *Entamoeba histolytica* from either monoxenic or axenic cultures. Here, the use of this technique for the isoenzyme separation of glucose phosphate isomerase (GPI) was studied for its ability to differentiate *Naegleria* species, including *N. fowleri*, grown with either *Escherichia coli* or in a semi-defined axenic broth medium.

3.3. Materials and Methods

3.3.1. Culture of amoebae. The strains of *Naegleria* spp. and *W. magna* studied are given in Table II. Axenic trophozoites were maintained at 32°C, or room temperature for *N. jadini*, in #SCGYM (**Appendix 1.2**). Strains were also grown in ¼ strength Ringer's solution containing a turbid suspension of live or heat-killed *E. coli* (strain JM101; 65°C for 15 minutes).

3.3.2. Preparation of amoebal lysates. Precise details for the preparation of trophozoite lysates, CAME and GPI detection is given in **Appendix 2.1 - 2.1.1 and 3.1**. Briefly, trophozoites were pelleted by centrifugation at 500 x g for 5 minutes, washed with ¼ strength Ringer's solution, and lysed in Dh_2O-0.2% Triton X-100 (v/v). Following centrifugation at 13,500 rpm for 1 minute, the supernatants were used immediately for CAME.

3.3.3. CAME and GPI detection. The electrode buffer consisted of 100 mM tris(hydroxymethyl)aminomethane (Tris), 100 mM maleic acid, 20 mM $MgCl_2$ and 10 mM disodium ethylenediamine tetracetic acid (EDTA), adjusted to pH 7.4. The membrane buffer was a 1:15 dilution of the electrode buffer.

Electrophoresis membranes (Titan III iso-vis mylar backed cellulose acetate membranes) and sample application system (Helena Super CPK system) were obtained from Helena Laboratories, Beaumont, Texas, USA and used according to the manufacturer's instructions. The system consists of an 8-well sample plate, an aligning base for positioning the membrane and an applicator for simultaneous delivery of 0.5 $\mu\ell$ samples to the membrane.

Each membrane was submerged in membrane buffer, with care taken to avoid trapping air-bubbles in the cellulose acetate, left for 10 minutes and then gently blotted to remove excess buffer. Lysates were applied immediately with two sample applications for each lysate. Electrophoresis was performed using a standard horizontal flat-bed electrophoresis tank with filter paper wicks for contact between the electrode buffer and membrane. Separation was achieved using a constant current of 12 mA for 15 minutes. GPI migration was from cathode (-) to anode (+).

GPI was detected using fructose-6-phosphate; glucose-6-phosphate dehydrogenase; ß-NADP and tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue (MTT); phenazine methasulphate [PMS]). The reaction was stopped by submerging the membrane in 5% (v/v) acetic acid followed by washing in running tap water for 5 minutes. Because the intensity of the developed bands fades with time, the membranes were either photographed, or photocopied on to paper for a permanent record.

3.4. Results

The GPI profiles obtained by CAME with the strains of *Naegleria* spp. and *W. magna* examined in this study are shown in Figure 11 and Figure 12. A single GPI band was obtained for all the species, the relative mobility of which enabled *N. fowleri*, *N. lovaniensis*, *N. australiensis*, *N. andersoni* and *W. magna* to be differentiated. Discrimination of the sub-species *N. australiensis italica* and *N. andersoni jamiesoni* was also possible since their GPI bands migrate slightly behind or ahead of their corresponding species. *N. jadini* and *N. australiensis italica* were found to have very similar GPI band mobilities.

No intraspecies variation in GPI mobility was detected within the nine strains of *N. fowleri*, five of *N. lovaniensis*, seven of *N. australiensis* and three of *W. magna*. Repeating the tests using different lysate preparations, gave identical findings (results not shown). Similarly, no variation in GPI profiles was observed between strains cultured with live or heat-killed *E. coli* or in axenic broth medium. An additional GPI band, of bacterial origin, was observed with strains prepared with live *E. coli* cultures. This migrated well ahead of the amoebal bands and did not confuse the interpretation of the results (Figure 12). No GPI activity could be detected with lysates prepared with heat-killed *E. coli*, showing that the enzyme had been inactivated.

To test the sensitivity of the method, 50 $\mu\ell$ lysates were prepared from two-fold dilutions of 1×10^6 trophozoites of *N. fowleri* (MCM), grown with heat-killed *E. coli*, and CAME of GPI performed. A faint but recordable band could be detected with lysates prepared from approximately 1.6×10^4 trophozoites (results not shown). In theory, as little as 3200 trophozoites prepared in a volume of 10 $\mu\ell$ of lysis buffer could be used for strain identification.

By preparing a lysate derived from pooled trophozoites of *N. fowleri*, *N. lovaniensis* and *N. australiensis* a single reference standard for the species was obtained (Figure 12). This is of benefit when screening environmental isolates, as 7 unknown strains per membrane can be analyzed. The standard was prepared by combining approximately 1×10^6 trophozoites of each species for lysis in a volume of 200 $\mu\ell$. Following centrifugation the clear lysate was divided into 10 $\mu\ell$ volumes and stored at -20°C.

3.5. Discussion

The presence of *N. fowleri* in warm aquatic sites presents a potential threat to human life from PAM. It is therefore important to identify such locations in the interests of public safety. Similarly, it is important to recognise other thermophilic *Naegleria* such as *N. lovaniensis* and *N. australiensis* as their presence in an environment suggests conditions suitable for the growth of *N. fowleri* (De Jonckheere, 1982b; Kilvington *et al*, 1991). This demands methods that reliably provide for the rapid identification of the organism soon after primary isolation. CAME of GPI is well suited for this purpose, enabling not only the identification

of *N. fowleri* but also the differentiation of other thermophilic *Naegleria* found in association in the environment. The preparation of lysates is simple and does not require multiple freeze-thaw cycles or sonication, enzyme stabilisers or long ultracentrifugation at 4°C (Visvesvara & Healy, 1980; Pernin, 1984; Moss *et al*, 1988). Sample preparation, electrophoresis and GPI detection can be completed in approximately 40 minutes. Several membranes can be run at one time, depending on the size of the electrophoresis chamber, enabling numerous strains to be examined.

Perhaps the greatest advantage of GPI CAME is that testing can be performed on strains grown with live or heat-killed *E.coli* thus eliminating the need for adaptation to axenic culture. This can take several weeks to achieve and is not always successful. No difference in the GPI profiles was found in strains of *Naegleria* spp and *W. magna* cultured on *E. coli* and then adapted to axenic culture. This is in contrast with the findings for *E. histolytica*, in which a change in zymodeme profile and pathogenic conversion was reported to have occurred in a strain adapted from bacterial to axenic culture (Mirelman *et al*, 1986).

All currently described species of *Naegleria* were included in this study except *N. gruberi*. Mainly by default, strains unable to grow above 37°C have been assigned to this species. However, recent isoenzyme studies using cellulose acetate gels have shown that *N. gruberi* is a heterogeneous group probably comprising several distinct species (Robinson *et al*, 1992). While the purpose of this study has been the development of a rapid and reliable method for differentiating *N. fowleri* from other thermophilic species, and not the investigation of *Naegleria* taxonomy, it would be of interest to establish whether CAME of GPI can also separate strains of *N. gruberi*. Although *N. jadini* and *N. australiensis italica* had very similar GPI mobilities the species are unlikely to be confused as their respective maximum temperature tolerances are 35°C and 42°C (Robinson *et al*, 1992). Strains of *W. magna* were included in this study because it is an amoeboflagellate, capable of growth at 45°C and is found in warm aquatic environments worldwide (De Jonckheere *et al*, 1984b). Although cyst morphology and the characteristically large trophozoite distinguishes the species from those of the *Naegleria*, confusion may arise which can be resolved by its unique CAME GPI profile.

The *N. australiensis* strains 4684.11, 5858.3 and 5858.5 used in this study were isolated from thermal spring water in Bath, England that was the source of a fatal case of PAM in 1978 (Cain *et al*, 1981; Kilvington *et al*, 1991). The identity of the isolates was first made by CAME of GPI and subsequently confirmed by antigenic analysis and pathogenicity for mice (De Jonckheere, 1981). These strains represent the first reported isolation of *N. australiensis* in the United Kingdom.

Table II. *Naegleria* and *W. magna* strains examined by CAME of GPI

Species	Strain	Origin	Source
N. fowleri	MCM	Bath, England (PAM)	a
	NF-3	Thermal springs, Bath, England	a
	158-44-3	Power station, Nottingham, England	a
	168-44-5	River water, Nottingham, England	a
	KUL	Belgium (PAM)	b
	HB-1	USA (PAM)	b
	NF-124	Thermal water, USA	b
	Carter 69	Australia (PAM)	b
	MSM	New Zealand (PAM)	c
N. lovaniensis	Aq/9/1/45D[1]	Aquarium, Belgium	b
	C-0490	Thermal springs, Bath, England	a
	EX5D/5	Hospital cooling tower, England	a
	HSP154	Hot springs, USA	d
	Ng 045	Water supply, Australia	e
N. australiensis	PP397[1]	Flood water, Australia	c
	4684.11	Thermal springs, Bath, England	a
	5858.3	Thermal springs, Bath, England	a
	5858.5	Thermal springs, Bath, England	a
	LSR34a	Thermal water, France	c
	PV2891	Thermal spa water, Italy	c
	NJ	Pond, India	c

Table II. *Naegleria* and *W. magna* strains examined by CAME of GPI

Species	Strain	Origin	Source
N. australiensis italica	AB-T-F_3[1]	Thermal spa water, Italy	b
N. andersoni	PPMFB-6[1]	Aquarium, Australia	b
N. andersoni jamiesoni	T56E[1]	Tropical fish import, Singapore	b
N. jadini	CCAP 1518/2[1]	Swimming pool, Belgium	g
W. magna	Z503[1]	Bovine faeces, France	b
	NI 13	Pond, India	f
	PAOB CL_4	Pond, Spain	f

PAM = from a case of primary amoebic meningoencephalitis

[1] denotes type strain of the species

Strains received from:

[a] S. Kilvington, Public Health Laboratory, Bath, England

[b] Dr J. De Jonckheere, Instituut voor Hygiene en Epidemiologie, Brussels, Belgium

[c] Dr D. Warhurst, London School of Hygiene and Tropical Medicine, London, England

[d] Dr W. O'Dell, Dept of Biology, University of Nebraska at Omaha, USA

[e] Mr P. Christy, State Water Laboratory, Salisbury, South Australia

[f] Dr R. Michel, Ernst-Rodenwald-Institut Medical Parasitologie, Koblenz, Germany

[g] Susan Brown, Culture Collection of Algae and Protozoa, Windermere, Cumbria, England

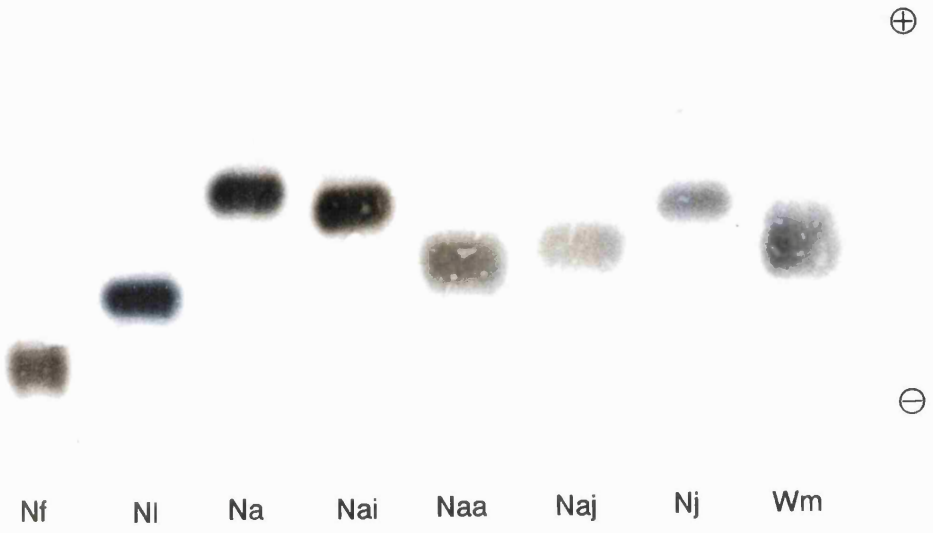

Figure 11 Differentiation of *N. fowleri* by CAME with respect to GPI

N. fowleri (Nf); *N. lovaniensis* (Nl); *N. australiensis* (Na); *N. australiensis italica* (Nai); *N. andersoni* (Naa); *N. andersoni jamiesoni* (Naj); *N. jadini* (Nj); and *W. magna* (Wm)

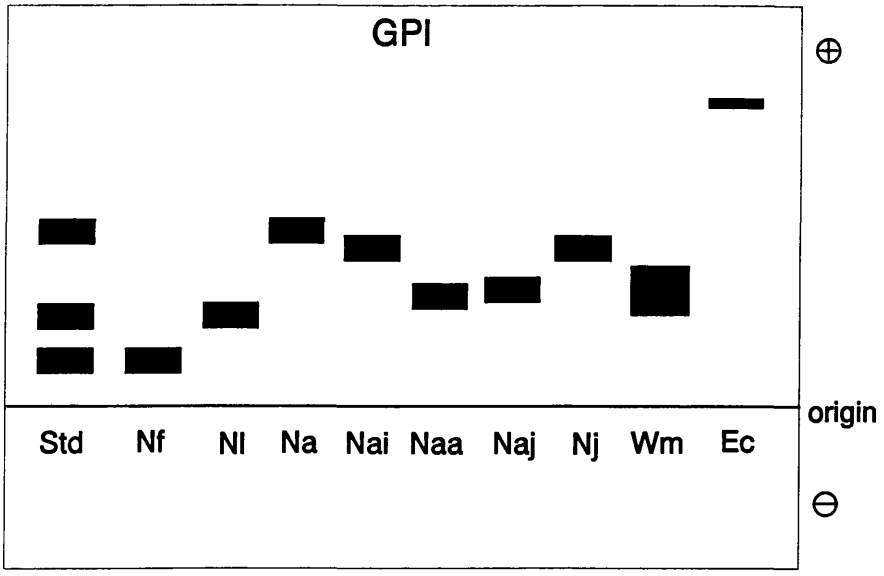

Figure 12 Diagram showing *Naegleria* spp. and *W.magna* CAME GPI mobilities

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Naegleria spp. and *W. magna* GPI isoenzyme profiles following CAME: reference standards from a combined *N. fowleri*, *N. lovaniensis* and *N. australiensis* lysate (Std); *N. fowleri* (Nf); *N. lovaniensis* (Nl); *N. australiensis* (Na); *N. australiensis italica* (Nai); *N. andersoni* (Naa); *N. andersoni jamiesoni* (Naj); *N. jadini* (Nj); *W. magna* (Wm) and *E. coli* (Ec)

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4. THE DEVELOPMENT OF DNA PROBES FOR THE IDENTIFICATION OF *NAEGLERIA FOWLERI*

4.1. Summary

A genomic library of *Naegleria fowleri* DNA was constructed in the λ phage vector EMBL3 and subclones made into the plasmid vector pUC 18. These were screened to identify clones that hybridised specifically to *N. fowleri* DNA. Two such clones, pB2.3 (a 1.2 kbp insert) and pB2.2.4 (a 0.8 kbp insert) were found to be suitable in this respect and in titration experiments could detect as little as 6.25 pg of *N. fowleri* DNA, equivalent to about 36 trophozoites. Restriction endonuclease digestion of whole-cell DNA enabled the detection of restriction fragment length polymorphisms (RFLPs) directly on agarose gel electrophoresis. As has been reported previously, this enabled strains of *N. fowleri* to be differentiated by their continent of origin. Isolates from the Europe showed RFLPs distinct from those from the Antipodes whilst strains from the USA were of either profile. However, here it was found that the USA strains of European profile could be uniquely characterised by RFLPs resulting from double digestion with the enzymes EcoR I and Hind III. A strain from Czechoslovakia was also found to have the distinctive EcoR I-Hind III RFLPs and indicates that the USA subgroup also occurs in Europe. A third pUC 18 subclone pB2.2 (a 6.1 kbp insert) was found to contain a repeated element that detected RFLPs of chromosomal origin that were not visible on agarose gel electrophoresis. This enabled the further differentiation of strains within the geographically defined whole-cell DNA RFLP groups.

4.2. Introduction

When monitoring environments for the presence of *N. fowleri* it is important to rapidly and reliably differentiate the species from other thermophilic *Naegleria*. A variety of techniques have been studied for this purpose including serological tests using species specific monoclonal antibodies (Visvesvara *et al*, 1987), isoenzyme electrophoretic profiles (De Jonckheere, 1982a; Kilvington *et al*, 1984; Pernin, 1984), characterisation of DNA restriction fragment length polymorphisms (RFLPs: De Jonckheere, 1987c; McLaughlin *et al*, 1988; Milligan & Band, 1988)) and the amplification of unique regions of *N. fowleri* DNA using the polymerase chain reaction (McLaughlin *et al*, 1991; Sparagano, 1993a & 1993b). The advantage of isoenzyme and whole-cell RFLP analysis is that they permit the simultaneous identification of all currently recognised species of *Naegleria*. The disadvantage is that they are expensive, time consuming and require large numbers of amoebae which usually have to be adapted to axenic culture. Although the use of cellulose acetate membrane electrophoresis with respect to glucose phosphate isomerase can be performed with *Naegleria* grown on NNA-*E. coli* (see *3. Identification of Naegleria species using cellulose acetate membrane electrophoresis of glucose phosphate isomerase*), the test is limited by the need to subculture strains following primary isolation, and also by the number of samples which can be analyzed on each membrane.

Genomic DNA is a highly complex structure in which specific sequences occur that uniquely characterise an organism. Advances in recombinant DNA now enable these regions to be isolated, manipulated and used as probes in the detection of homologous sequences in the rapid identification of organisms (Tenover, 1988; Sambrook *et al*, 1989; Barker, 1990; Girardin *et al*, 1993). A typical strategy for the preparation of a DNA probe to a particular organism usually comprises: (i) isolation of the organism DNA; (ii) digestion of the DNA with a restriction endonuclease to produce discrete fragments; (iii) cloning of the fragments into a plasmid or phage vector for amplification in a host bacterium; (iv) reisolation of the cloned, amplified DNA and analysis in hybridisation studies. Here, the cloned DNA is labelled by the incorporation of radioactive nucleotides such as 5'-[α-^{32}P] deoxycytidine triphosphate or a nonradioactive source, denatured to render it single stranded and then allowed to react with denatured, unlabelled DNA of other organisms which is usually immobilised on to a cellulose nitrate or nylon support membrane . The labelled DNA probe binds to any complementary DNA sequence on the membrane and after washing to remove unbound probe, is detected by autoradiography (if the probe is radio-labelled) or some other reaction appropriate to the labelling method used (e.g., immunoenzymatic detection: Wolcott, 1992). The key step in these procedures is to identify DNA probes which will detect DNA sequences in a given organism and not cross-react with others.

Such an approach has been used successfully to develop specific and sensitive DNA probes to a variety of microbes including environmental bacteria of the genus *Legionella* (Grimont *et al*, 1985; Mahbubani *et al*, 1990; Bej *et al*, 1991a) and pathogenic protozoa such as *Entamoeba histolytica* (Samualson *et al*, 1989; Tannich *et al*, 1989; Bracha *et al*, 1990), *Giardia intestinalis* ([*duodenalis*]: Nash *et al*, 1985; De Jonckheere *et al*, 1989; Mahbubani *et al*, 1992), *Trichomonas vaginalis* (Rubino *et al*, 1991; Paces *et al*, 1992) *Babesia bovis* (McLaughlin *et al*, 1986) and *Plasmodium falciparum* (Barker, 1990). Although DNA clones from *N. gruberi* and *N. fowleri* have been described, these cross-hybridised with other species and have been used only for taxonomic studies (Clark & Cross, 1987; Clark & Cross, 1988a; Clark *et al*, 1989). The object of this study was to isolate clones of *N. fowleri* DNA for use as probes in the development of a rapid and specific identification method for the organism soon after primary isolation from the environment.

4.3. PART I: THE DEVELOPMENT OF *N. FOWLERI* SPECIFIC DNA PROBES

4.3.1. Materials and Methods

4.3.1.1. Organisms studied. The organisms used in this study are given in Table III. The monkey kidney and human tissue culture cell lines were obtained from the virology department of the Bath Public Health Laboratory. Algal cultures were received from Dr John Wright, School of Biology and Biochemistry, University of Bath, England. *Legionella pneumophila* sg. 1 and *L. micdadei* DNA was obtained from Dr Norman Fry at the Central Public Health Laboratory, Colindale, London. Strains of *Naegleria* spp.,

Acanthamoeba spp. and *Willaertia magna* were maintained in #SCGYM or #YPNFH at 32°C (**Appendix 1.2 & 1.3**). Other FLA: *Hartmannella vermiformis*, *Vannella* spp. and *Vahlkampfia* sp. were grown on non-nutrient agar plates seeded with the bacterium *Klebsiella edwardsii* strain K10896 (**Appendix 1.1**). *Tetrahymena pyriformis* was grown in brain heart infusion broth at room temperature. *Trichomonas vaginalis* was grown in brain heart infusion broth supplemented with 0.1% cysteine hydrochloride, 0.02% ascorbic acid and 10% heat inactivated horse serum. *Pseudomonas aeruginosa*, *E. coli* and *K. edwardsii* were grown on LB agar (**Appendix 1.11**) in air at 37°C.

4.3.1.2. DNA isolation. The protocol for isolating whole-cell DNA is given in **Appendix 3.2**. Briefly, for axenic cultures of FLA, *T. pyriformis*, *T. vaginalis*, human white blood cells, tissue culture cells and algae approximately 1 x 10^8 late log phase cell cultures were harvested by centrifugation at 500 x g for 10 minutes at room temperature, washed once with phosphate buffered saline (PBS) and resuspended in cell lysis buffer. For FLA growing on NNA-*K. edwardsii*, approximately 1 x 10^7 trophozoites were washed from the plates with ice-cold PBS, centrifuged at 500 x g for 10 minutes, washed once with PBS and resuspended in cell lysis buffer. For the bacteria strains, 10 colonies were picked directly into cell lysis buffer.

Whole-cell DNA was isolated by adding proteinase K (10 mg/ml) and sarcosyl (20%) to a final concentration of 150 μg/ml and 2% respectively and incubated at 56°C for 4 hours or overnight with occasional mixing. The lysate was extracted once with phenol:chloroform:isoamyl alcohol ("phenol") and once with chloroform:isoamyl alcohol ("chloroform"). These steps were facilitated by use of SST blood collection tubes (Becton Dickinson) that contain a silicone gel material which forms a barrier between the organic and aqueous layers after centrifugation. The denatured protein is also trapped below the gel barrier with the organic layer. The upper aqueous phase containing the nucleic acids can then simply be poured off into a fresh tube. Nucleic acids were precipitated with an equal volume of iso-propanol and washed twice with 70% ethanol. After briefly drying, the pellet was dissolved in TE Buffer (pH 8.0) containing 25 μg/ml RNase A and stored at -20°C. DNA concentrations were estimated by comparison with standards spotted on to agarose plates containing the dye Hoechst H33258 with UV illumination (Rieber & Rieber, 1990) as described in **Appendix 3.5**.

4.3.1.3. Cloning of *N. fowleri* (MCM) DNA in the λ phage vector EMBL3. EMBL3 is a lambda (λ) phage replacement vector capable of accepting BamH I compatible fragments (e.g., Sau3A I, Mbo I, Bgl II or BamH I) ranging in size from 9 to 23 kbp (Figure 13). The arms are prepared by double digestion with BamH I and EcoR I followed by selective precipitation, which removes the small BamH I-EcoR I linker that separates the arms from the central stuffer fragment. DNA for cloning is size fractionated by partial restriction endonuclease digestion and ligated with the EMBL3 arms. It is usual to phosphatase treat the target DNA to be cloned to prevent self-annealing between small DNA fragments with compatible ends which can produce concatamers of sufficient size to be cloned into EMBL3. The ligated λ phage is then packaged and absorbed to a suitable *E. coli* host strain. Following plating on to a nutrient agar medium and

incubation to allow the growth of the *E. coli*, the λ phage infection is seen as discrete areas of lysis, termed plaques, in the bacterial lawn as shown in Figure 14.

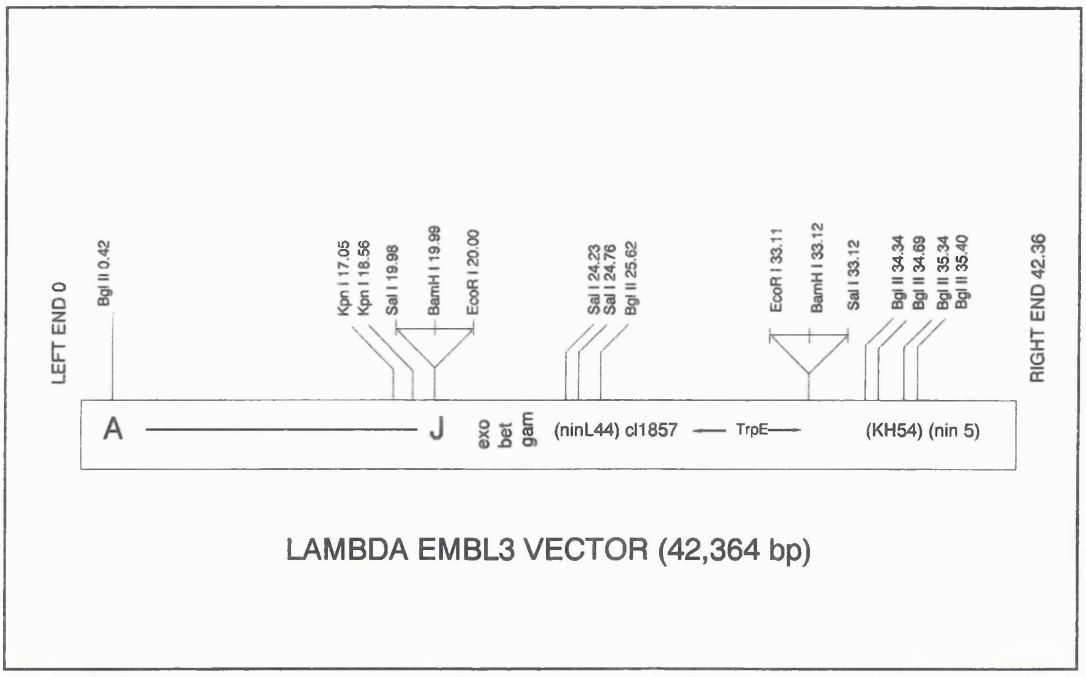


Figure 13 Genomic structure of the λ phage vector EMBL3

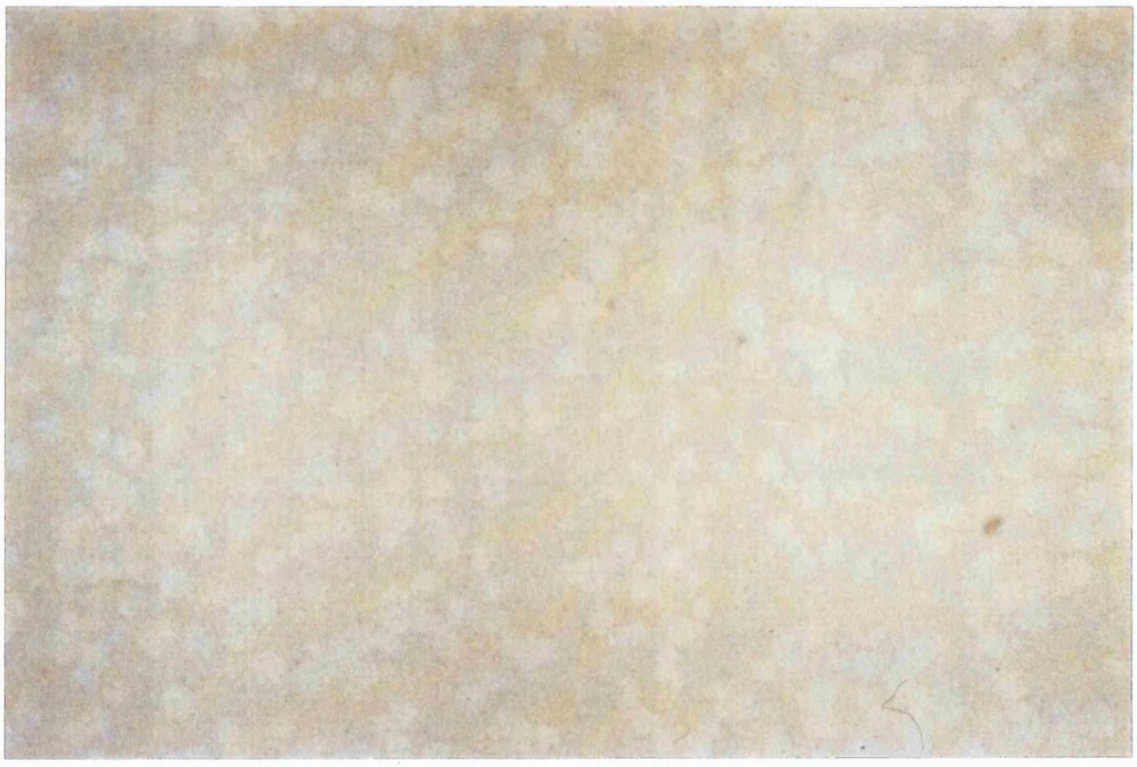


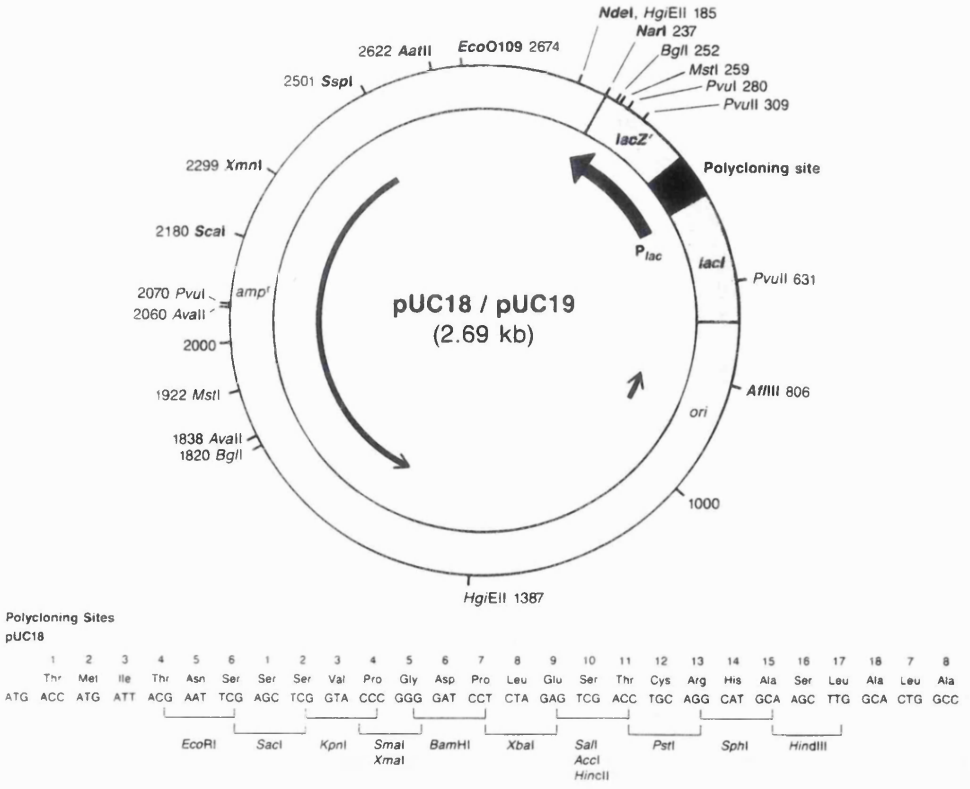
Figure 14 λ phage plaques on a lawn of *E. coli*

Detailed protocols for the preparation of size fractioned *N. fowleri* DNA, ligation into EMBL3, packaging of recombinant phage, infection of *E. coli* and plating of cells is given in **Appendix 3.6 - 3.10.1**. Briefly, *N. fowleri* (MCM) DNA was partially digested with Sau3A I (Northumbria Biologicals Ltd, Northumbria, England) to produce fragments ranging from 15-22 kbp as determined by electrophoresis on a 0.5% agarose gel. Following dephosphorylation with calf intestinal alkaline phosphatase, 300 ng of DNA was ligated with 1 μg of the EMBL3 λ phage vector (Stratagene, Cambridge, England). Ligation was performed for 1 hour at 22°C followed by 48 hours at 4°C with 4 U of T4 DNA ligase in the buffer supplied by the manufacturer (Northumbria Biologicals Ltd, Northumbria, England). Recombinant phages were packaged using Gigapack™ II Plus packaging extract (Stratagene, Cambridge, England) and used to infect *E. coli* strain P2392. Dilutions of the cells were made in phage dilution buffer and plated on to NZY agar for incubation at 37°C overnight. Appropriate controls of EMBL3 arms without *N. fowleri* DNA and with a ~12 kbp test insert supplied with the vector were also ligated, packaged and plated as described above.

4.3.1.4. Analysis of recombinant phage clones. Plaque lifts from NZY agar plates were made in duplicate on to Hybond N nylon membranes (Amersham, Buckinghamshire, England) as described in **Appendix 3.10.2**. Membranes were hybridised with either whole-cell DNA from *N. fowleri* (MCM) or *N. lovaniensis* (C-0490) that had been sheared by forcing several times through an 18 g hypodermic needle and syringe and labelled with 5'-[α-^{32}P] deoxycytidine triphosphate by the random primer or oligolabelling method (Feinberg & Vogelstein, 1983: **Appendix 3.11 - 3.12**). Plaques reacting strongly with the *N. fowleri* whole-cell DNA probe and weakly or not at all with that of *N. lovaniensis* were picked, replated and tested again for hybridisation with labelled *N. fowleri* whole-cell DNA (**Appendix 3.13.**).

Small and large scale λ phage preparations of the selected recombinant clones were made from NZY plate lysates as described in **Appendix 3.14**. Cloned insert DNA was released by digestion with Sal I (restriction sites for this enzyme flank the BamH I-Eco RI cloning region) and analyzed by electrophoresis in 0.7% agarose TBE gels (**Appendix 3.3 - 3.4**).

4.3.1.5. Subcloning into pUC 18 plasmid vector. The pUC series of plasmids were developed by Joachim Messing and colleagues in the 1980's. These plasmids carry a segment of the *lac* operon from *E. coli* that encodes for the production of the enzyme β-galactosidase (Figure 15). Synthesis is induced by isopropylthio-β-D-galactoside (IPTG) and the fragment produced undergoes intra-allelic (α) complementation with a defective form of β-galactosidase encoded by the host bacterium (Sambrook *et al*, 1989). When plated on to culture media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) bacteria containing the plasmid and exposed to IPTG give rise to blue colonies. If, however, foreign DNA is inserted into the region of the *lac* operon, the production of β-galactosidase is halted and bacteria containing the recombinant plasmid give rise to white colonies on X-gal media (Figure 16). The plasmid also contains the gene for ampicillin resistance and when used in conjunction with a normally sensitive *E. coli* host strain, only bacteria containing the plasmid will grow on media containing the antibiotic.



Polycloning Sites
pUC18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
						EcoRI		SacI		KpnI		SmaI XmaI		BamHI		XbaI		SalI AccI HincII		PstI		SphI		HindIII		

Figure 15 Plasmid vector pUC 18

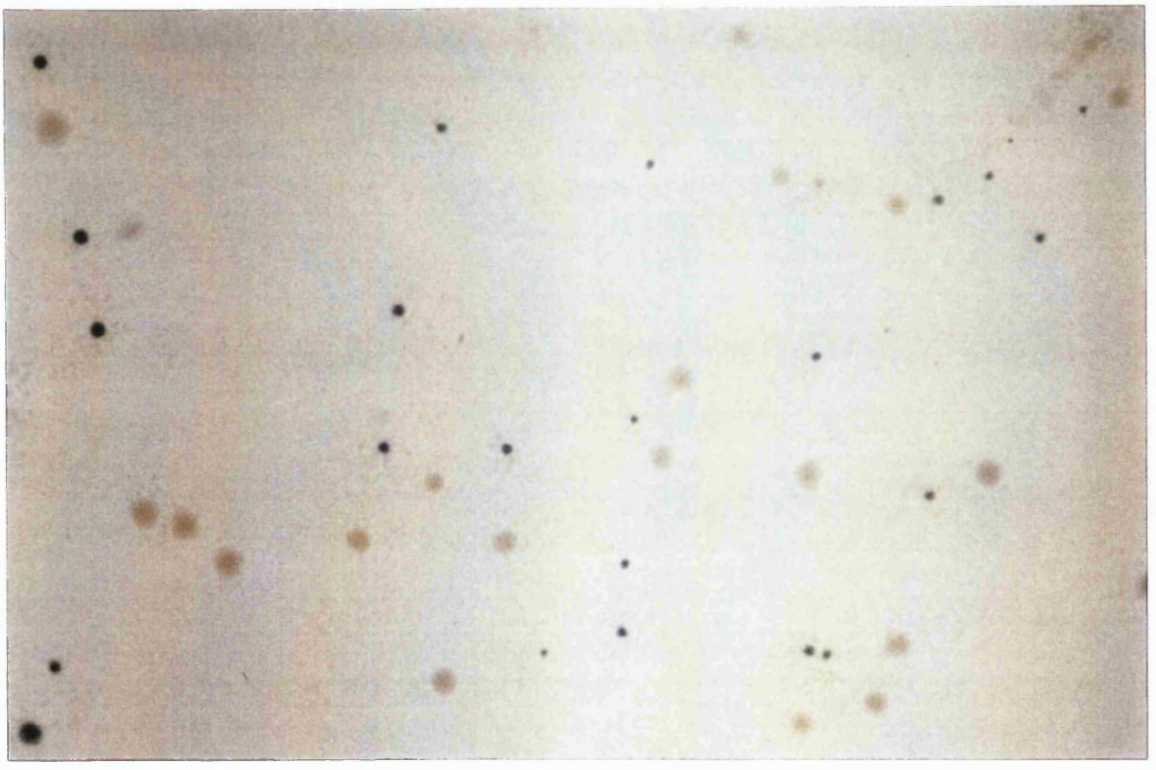


Figure 16 Transformed *E. coli* grown on IPTG and x-gal agar

The λ phage clones were digested with Sal I and separated on 0.7% agarose TAE gels (**Appendix 3.3 - 3.4**). DNA bands were cut from the gels and purified by absorption on to silica particles as described in **Appendix 3.15**. Briefly, agarose prepared in TAE buffer dissolves in aqueous sodium iodide at a final concentration of >1 M. Silica particles are then added to which the released DNA binds in the presence of the high salt concentration of sodium iodide. The bound DNA is then washed several times with a sodium chloride-ethanol solution by centrifugation and finally eluted from the silica in dH_2O or TE buffer.

The plasmid vector pUC 18 was linearised with Sal I and dephosphorylated with calf intestinal alkaline phosphatase to prevent self-ligation. Purified DNA was ligated into pUC 18 and used to transform of *E. coli* JM101 using the polyethyleneglycol-DMSO method described by Chung and colleagues (1989) as detailed in **Appendix 3.16 - 3.17**. As a control, Sal I digested and alkaline phosphatase treated pUC 18 plasmid was ligated alone. Recombinant cells were identified by X-gal-IPTG selection on ampicillin containing LB agar culture plates. Small and large scale plasmid preparations from recombinant *E. coli* cells were made using the alkaline lysis method (Sambrook *et al*, 1989; **Appendix 3.18**).

4.3.1.6. Southern hybridisation analysis of DNA. Approximately 3 μg of whole-cell *Naegleria* DNA was digested with 10-20 U of restriction endonucleases for 4-18 hours at 37°C using appropriate reaction buffers provided with the enzymes (Northumbria Biologicals Limited, Northumbria, England) as described in **Appendix 3.3**. Samples were loaded on to horizontal 0.7% agarose gels (20 cm x 20 cm x 0.5 cm) prepared in TBE buffer (**Appendix 3.4**). DNA standards of λ-Hind III/ΦX-174 RF-Hinc III digests (Pharmacia LKB Ltd, Milton Keynes, England) were included as size markers. Gels were stained with 1 μg/ml ethidium bromide in dH_2O for 1 h, destained in dH_2O for 20 minutes and photographed under shortwave UV transillumination using Polaroid 665 film and a Kodak Wratten #23A orange filter.

Gels were blotted on to Hybond N nylon membranes by the alkaline transfer method (Reed & Mann, 1985) according to the manufacturer's protocol detailed in **Appendix 3.19**. Membranes were placed DNA side down on to a transilluminator and exposed for 5 minutes to fix the DNA.

N. fowleri recombinant pUC 18 clones were digested with Sal I, separated on 0.7% agarose TAE gels and the appropriate DNA fragments purified by absorption on to silica particles as described above. The DNA was then labelled with 5'-[α-^{32}P] deoxycytidine triphosphate by the oligolabelling method of Feinberg and Vogelstein (1983) as detailed in **Appendix 3.11**.

Prehybridisation and hybridisation of membranes was performed at 65°C in "Westneat buffer" (Westneat *et al*, 1988) as described in **Appendix 3.12**. This method uses 7% SDS and 1% BSA as blocking agents to prevent nonspecific binding in the hybridisation reaction. Following washing at room temperature for 2 x 15 minutes in 2XSSC-0.1% SDS; 15 minutes in 2XSSC-0.1% SDS at 65°C; 30 minutes in 1XSSC at 65°C and 30 minutes in 0.1XSSC at 65°C the membranes were exposed on to Fuji RX medical X-ray film in autoradiography cassettes (Genetic Research Instrumentation Ltd) with intensifying

screens. Typical exposure times were 3-5 days at -70°C. Exposed film was developed in Kodak D19 developer for 5 minutes, rinsed briefly in tap water, fixed in Kodak 'Unifix' for 5 minutes and finally rinsed in tap water for 15 minutes and air-dried.

4.3.1.7. Dot-blot analysis of DNA. Purified DNA from the organisms listed in Table III was adjusted to a concentration of ~500 ng in 0.4 N NaOH-10 mM EDTA, heated at 70°C for 15 minutes and chilled on ice. The denatured DNA was then immobilised on to Hybond N nylon membranes using a commercial dot-blotting apparatus (Bio-Rad), air-dried and exposed, DNA side down, on a transilluminator for 5 minutes (**Appendix 3.20**. Membranes were hybridised with *N. fowleri* pUC 18 subclones labelled with 5'-[α-^{32}P] deoxycytidine triphosphate as described above. *N. fowleri* (MCM) DNA dilutions ranging from 1.6 ng to 1.6 pg were also immobilised on to membranes and used to test the sensitivity of detection by the probes.

N. fowleri (MCM) DNA was also extracted by the alkaline lysis method used for the isolation of *E. coli* plasmid DNA (**Appendix 3.18**) and tested in the dot-blot studies. This method selectively recovers mitochondrial and ribosomal DNA but not chromosomal DNA.

4.4. Results

The effects of limiting the concentration of Sau3A I on the digestion of *N. fowleri* (MCM) DNA is shown in Figure 17. From this it was estimated that 0.02 units /μg DNA of Sau3A I incubated at 37°C for 1 hour would give the maximum amount of digested DNA of size 15-22 kbp for ligation into EMBL3 λ phage vector (Figure 17). In the subsequent preparative digestions, two reactions were performed using 0.02 and 0.01 U of Sau3A 1 /μg of DNA. This was designed to increase the chances of obtaining the maximum number of molecules within the range 15-22 kbp (Sambrook *et al*, 1989).

Replicate plaque lifts on to nylon membranes from the primary plating of the *N. fowleri* λ phage library were hybridised with 5'-[α-^{32}P] deoxycytidine triphosphate labelled whole-cell *N. fowleri* or *N. lovaniensis* DNA and compared (Figure 18). Twelve clones which reacted strongly with the *N. fowleri* probe but only weakly or not at all with that from *N. lovaniensis* were picked, replated and screened again for specificity with the two probes.

The efficiency of packaging in the construction of the λ phage library was calculated by:

$$\frac{(\text{Nº of plaques}) \times (\text{dilution factor}) \times (\text{total packaging volume } 500\ \mu\ell)}{(\text{amount DNA [in } \mu\text{g] packaged}) \times (\text{Nº of } \mu\ell \text{ plated})}$$

This gave a value of 2.5 x 10^6 plaque forming units (pfu) /μg of *N. fowleri* (MCM) DNA indicating that the process occurred with high efficiency. The control experiments in which the EMBL3 arms were ligated and packaged without *N. fowleri* DNA and with a ~12 kbp test insert supplied with the vector gave figures of 4 x 10^4 and 3.8 x 10^6 respectively.

To establish how representative of the *N. fowleri* genome was the library, the following formula was applied:

$$N = \frac{\ln (1-p)}{\ln (1-x/y)}$$

Where p = 0.99; x = size of cloned insert DNA (assumed to average 20 kbp); y = size of genome of *N. fowleri* (140,000 kbp).

In this case a value of 3.2 x 10^4 was obtained. Therefore, to have ensured the complete cloning of the *N. fowleri* genome in a series of 20 kbp fragments would require 3.2 x 10^4 pfu /μg of DNA. As the library constructed here yielded 2.5 x 10^6 pfu, it was considered to be highly representative of the entire *N. fowleri* genome.

The clones from the screened λ phage library were amplified and small scale λ phage DNA preparations made. Digestion with Sal I or Sal I together with EcoR I showed the size of the cloned DNA ranged from approximately 16 - 22 kbp (Figure 19). Two λ phage clones, which had internal Sal I restriction sites were selected for subcloning into pUC 18 plasmid vector. This gave rise to the subclones pB2.2, a 6.1 kbp fragment, and pB2.3, a 1.2 kbp fragment (Figure 20). pB2.2 was found to have an internal EcoR I restriction site which gave rise to a 0.8 kbp fragment. This also was cloned into the EcoR I site in pUC 18 and gave rise to the clone pB2.2.4 (Figure 20).

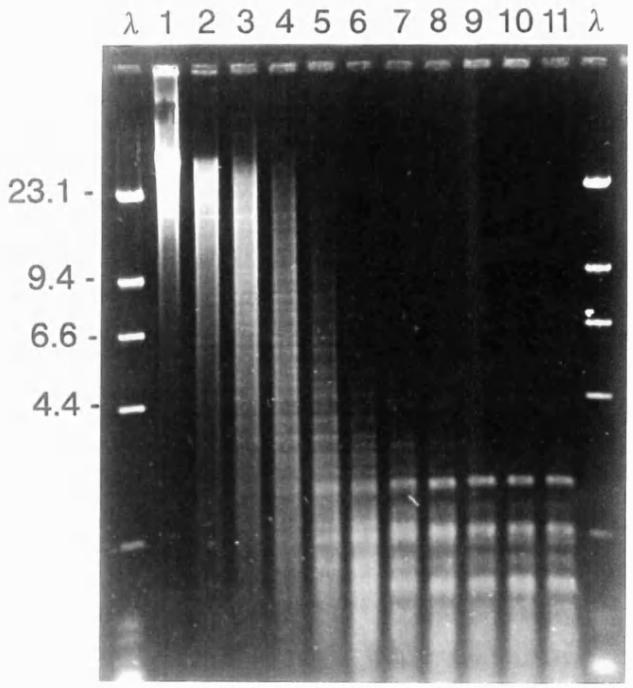


Figure 17 Sau3A I partial digestion of *N. fowleri* (MCM) DNA

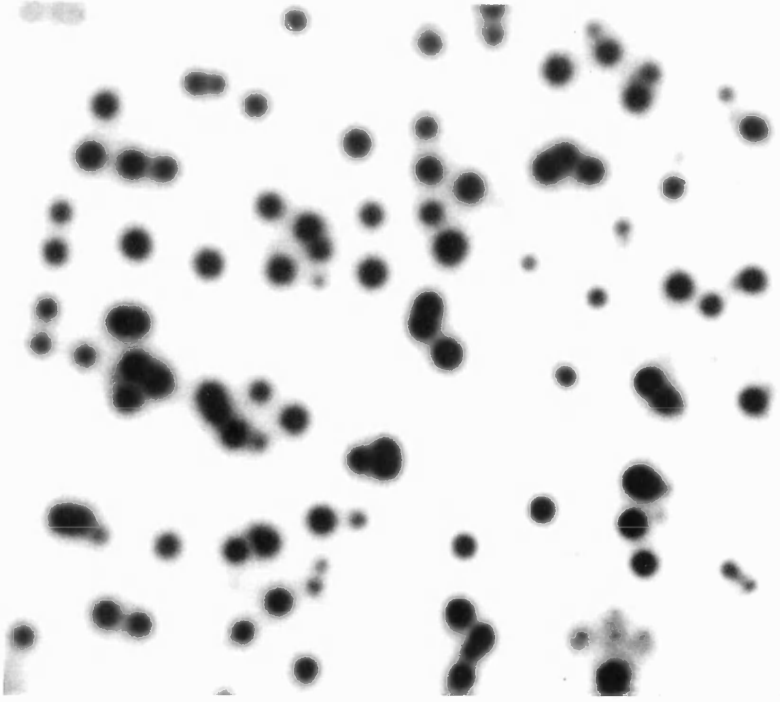


Figure 18 λ phage EMBL3 plaques hybridised with a *N. fowleri* (MCM) whole-cell DNA probe

Figure 19 Purified EMBL3 λ phage clones digested with Sal I or Sal I + EcoR I

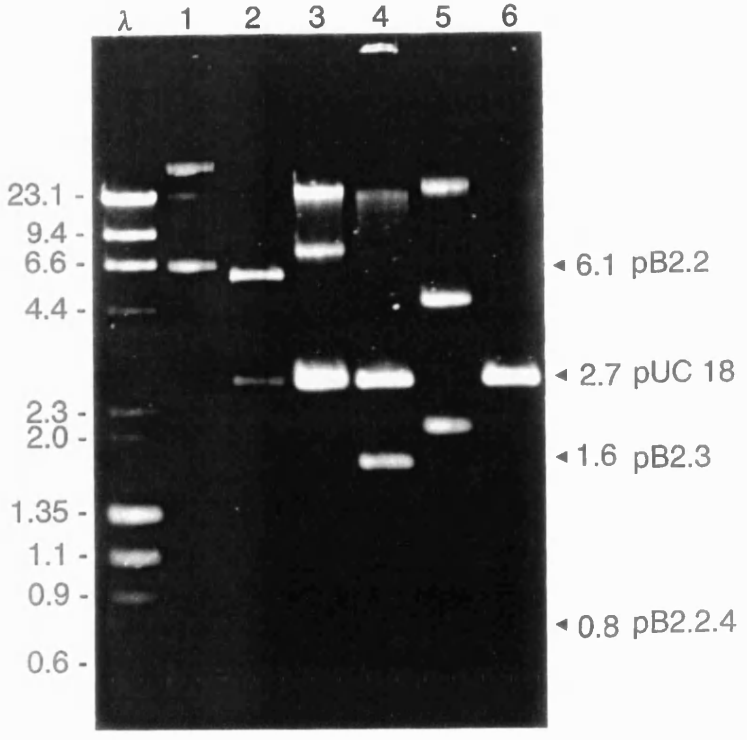


Figure 20 Purified pUC 18 subclones digested with Sal I or EcoR I

N. fowleri and *N. lovaniensis* whole-cell DNA preparations digested with EcoR I, Hind III or EcoR I-Hind III enabled the detection of numerous RFLPs directly on agarose gel electrophoresis (Figure 21, Figure 25 and Figure 29). All the strains of *N. lovaniensis* gave homologous EcoR I, Hind III and EcoR I-Hind III RFLPs. These were quite different from those found with *N. fowleri* and readily enabled the differentiation of the two species. In contrast to the findings with *N. lovaniensis*, significant variation in agarose gel RFLPs were detected between *N. fowleri* strains and these corresponded to the geographic origin of the strains. With EcoR I or Hind III, strains from Europe were distinguished from those of the Antipodes whilst isolates from the USA were of either profile. However, with EcoR I-Hind III digestion the USA strains (6088 and NF-124) that showed RFLPs similar to those from the Antipodes were found to have a unique doublet of ~7-7.5 kbp (Figure 29). This was profile was also found with strain NF 59 from Czechoslovakia.

Minor interstrain variations were also detected within the geographically defined groups for *N. fowleri*. With Hind III, strain 6088 from the USA had an additional band of ~2.5 kbp (Figure 25). Strain MSM from New Zealand showed differences with EcoR I and EcoR I-Hind III by the presence of a ~6.2 kbp band in both cases (Figure 21 and Figure 29).

All three pUC 18 clones pB2.2, pB2.3, pB2.2.4 , hybridised specifically to membrane transfers of *N. fowleri* DNA digested with EcoR I, Hind III or EcoR I-Hind III (Figure 21, Figure 22, Figure 23, Figure 24, Figure 26, Figure 27, Figure 28, Figure 30, Figure 31, Figure 32). The probes pB2.3 and pB2.2.4 detected a single RFLP, common to all strains, both of size ~1.6 kbp for EcoR I, ~2.2 kbp for Hind III and ~1 kbp and <0.8 kbp respectively for EcoR I-Hind III. In contrast probe pB2.2 detected numerous RFLPs indicating it to be a clone of a repeated element within the *N. fowleri* genome. Overall, the pB2.2 RFLPs were similar for all the strains although some minor variations were detected. With Hind III, all the Antipodean strains had an additional ~3 kbp RFLP. With EcoR I-Hind III digests all the Antipodean strains, except MSM, lacked a ~3.4 kbp band common to those from the USA and Europe.

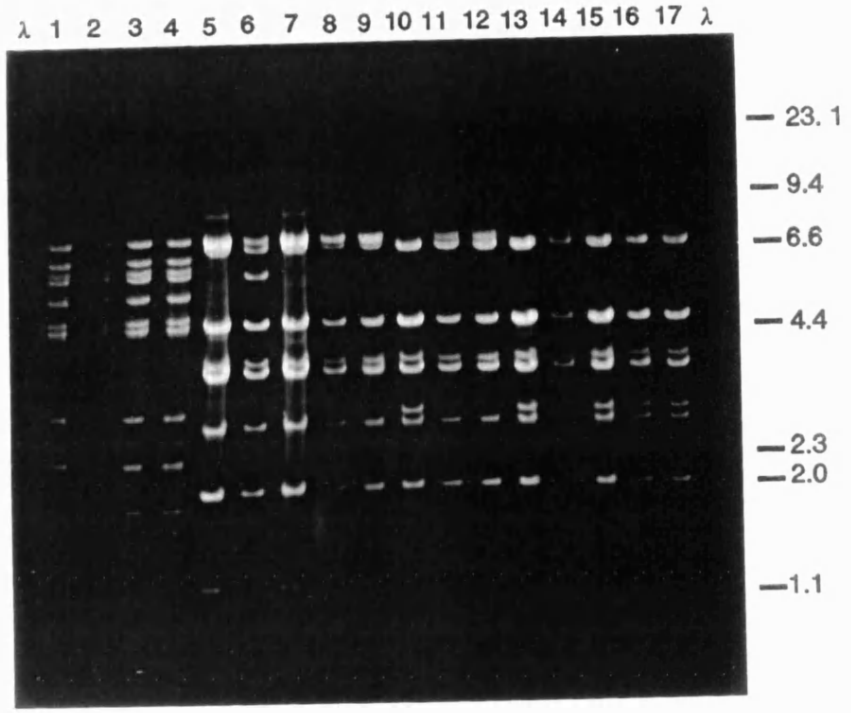


Figure 21 *N. lovaniensis & N. fowleri* EcoR I RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hinc III digest. *N. lovaniensis:* (1) HSP 154, (2) Aq/9/1/45D, (3) EX5D/25, (4) C-0490. *N. fowleri:* (5) NHI, (6) MSM, (7) 1518/4, (8) 1518/3, (9) Carter 69, (10) CDC:0487:1, (11) 6088, (12) NF-124, (13) HB-1, (14) NF-59, (15) KUL, (16) NF-3, (17) MCM

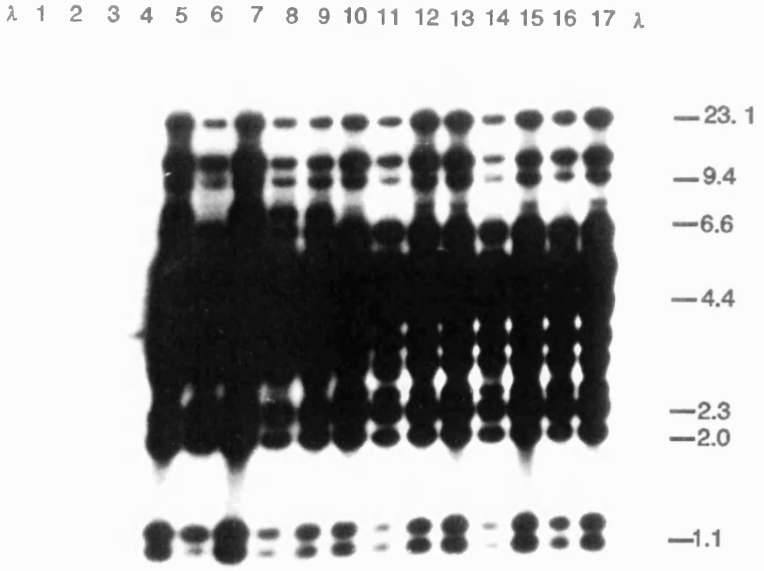


Figure 22 *N. lovaniensis & N. fowleri* EcoR I digests probed with pB2.2

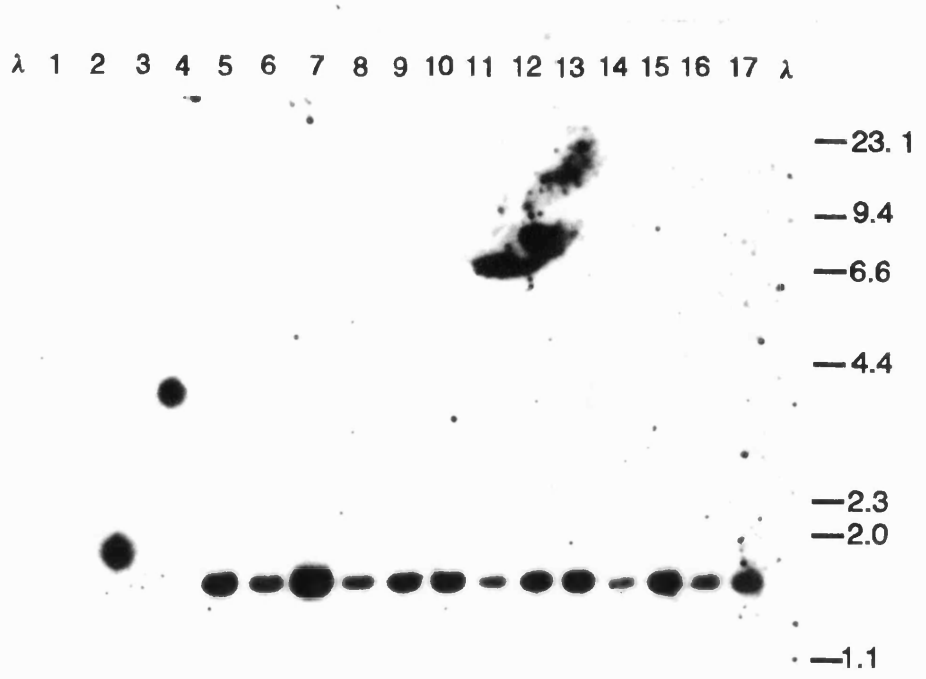


Figure 23 *N. lovaniensis* & *N. fowleri* EcoR I digests probed with pB2.3

(λ) lambda-Hind III/ΦX-174 RF-Hinc III digest. *N. lovaniensis:* (1) HSP 154, (2) Aq/9/1/45D, (3) EX5D/25, (4) C-0490. *N. fowleri:* (5) NHI, (6) MSM, (7) 1518/4, (8) 1518/3, (9) Carter 69, (10) CDC:0487:1, (11) 6088 , (12) NF-124, (13) HB-1, (14) NF-59, (15) KUL, (16) NF-3 (17) MCM

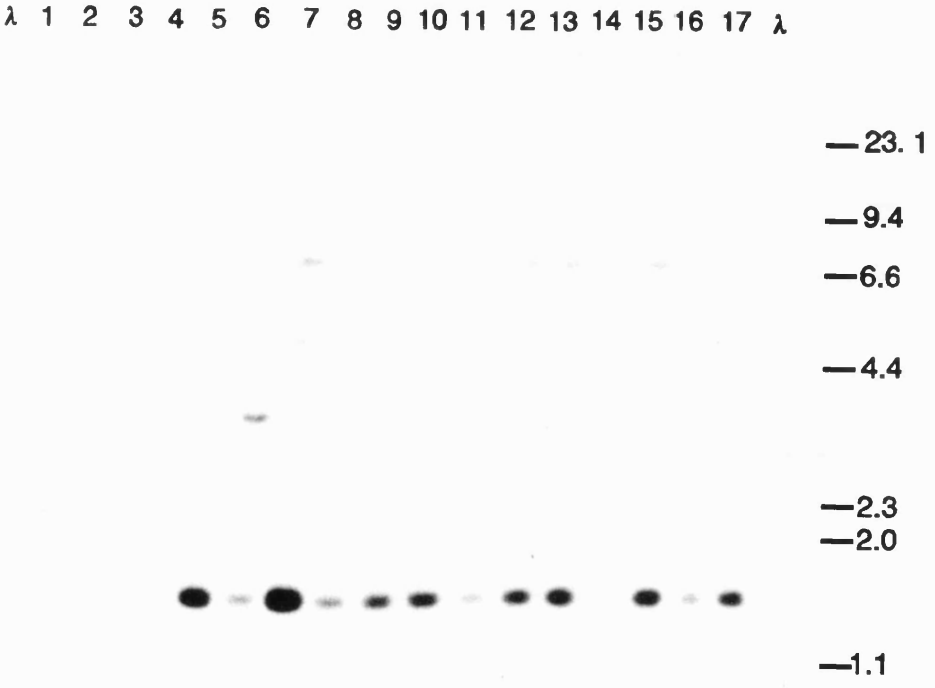


Figure 24 *N. lovaniensis* & *N. fowleri* EcoR I digests probed with pB2.2.4

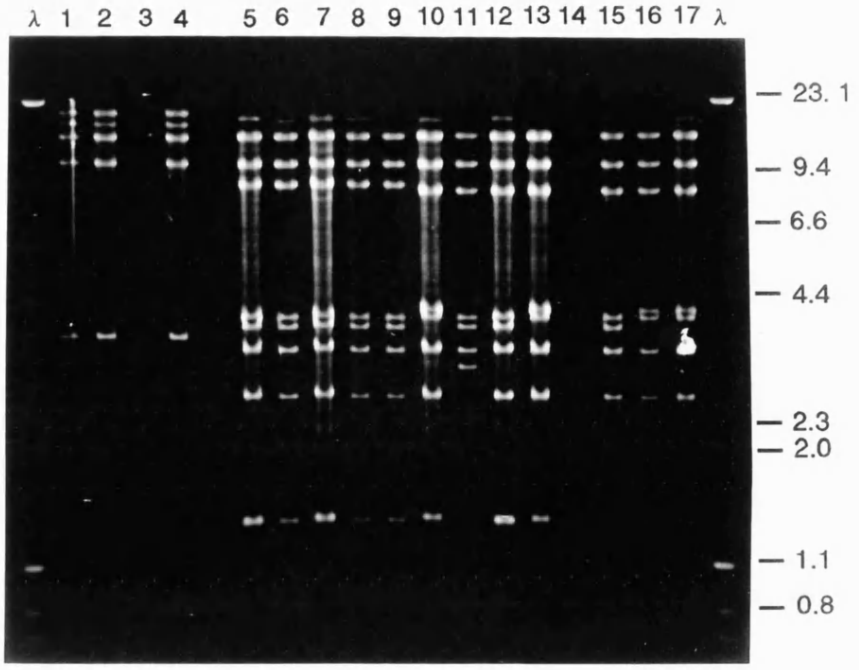


Figure 25 *N. lovaniensis* & *N. fowleri* Hind III RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hinc III digest. *N. lovaniensis:* (1) HSP 154, (2) Aq/9/1/45D, (3) EX5D/25, (4) C-0490. *N. fowleri:* (5) NHI, (6) MSM, (7) 1518/4, (8) 1518/3, (9) Carter 69, (10) CDC:0487:1, (11) 6088 , (12) NF-124, (13) HB-1, (14) NF-59, (15) KUL, (16) NF-3 (17) MCM

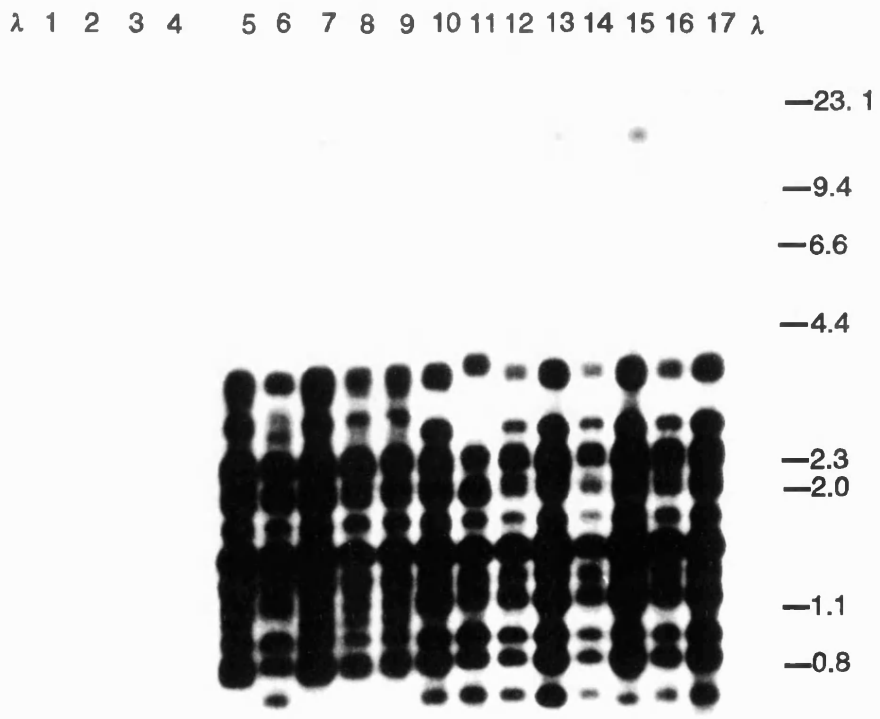


Figure 26 *N. lovaniensis* & *N. fowleri* Hind III digests probed with pB2.2

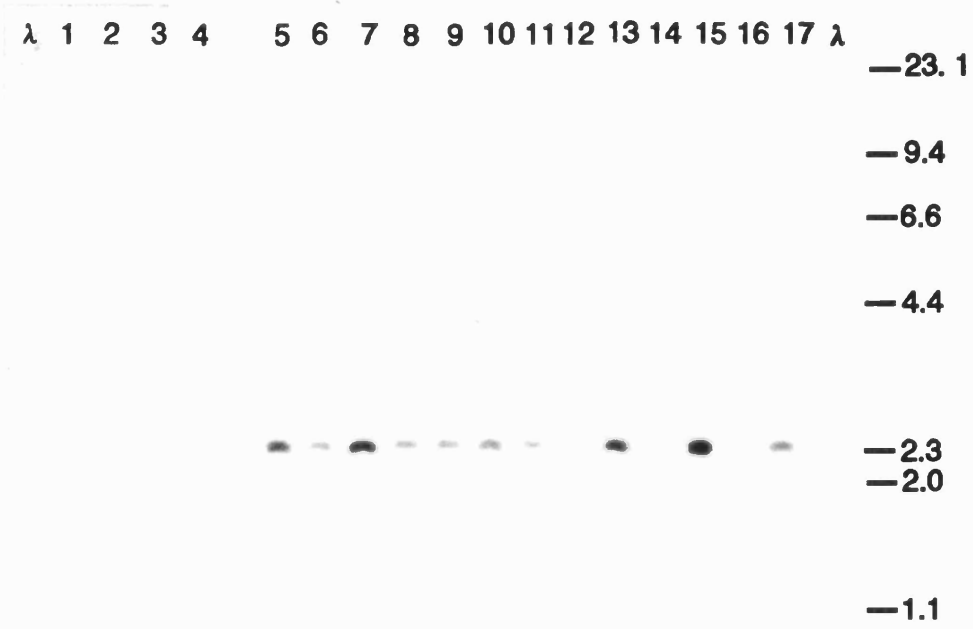


Figure 27 *N. lovaniensis* & *N. fowleri* Hind III digests probed with pB2.3

(λ) lambda-Hind III/ΦX-174 RF-Hinc III digest. *N. lovaniensis:* (1) HSP 154, (2) Aq/9/1/45D, (3) EX5D/25, (4) C-0490. *N. fowleri:* (5) NHI, (6) MSM, (7) 1518/4, (8) 1518/3, (9) Carter 69, (10) CDC:0487:1, (11) 6088 , (12) NF-124, (13) HB-1, (14) NF-59, (15) KUL, (16) NF-3 (17) MCM

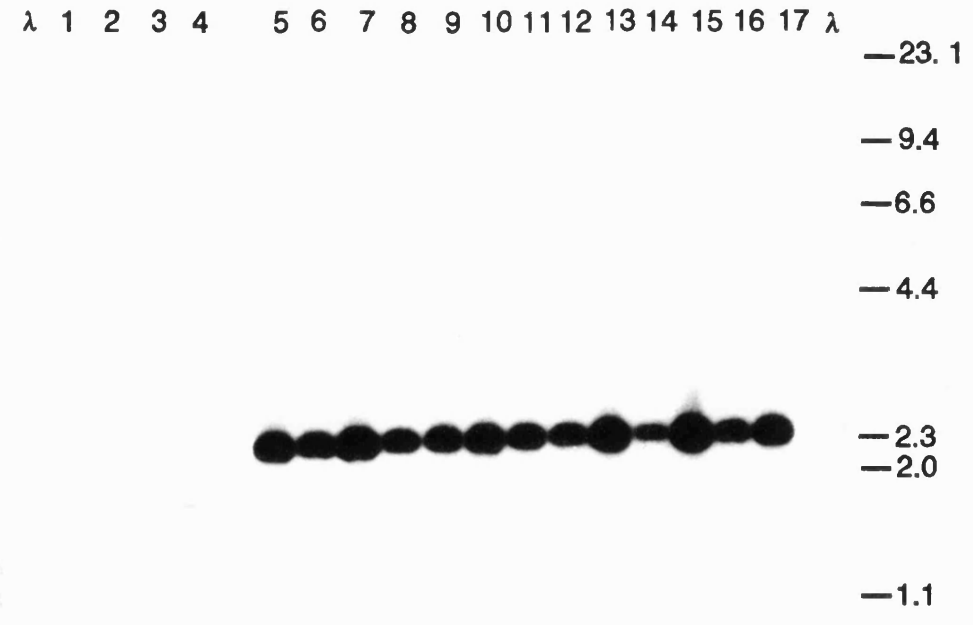


Figure 28 *N. lovaniensis* & *N. fowleri* Hind III digests probed with pB2.2.4

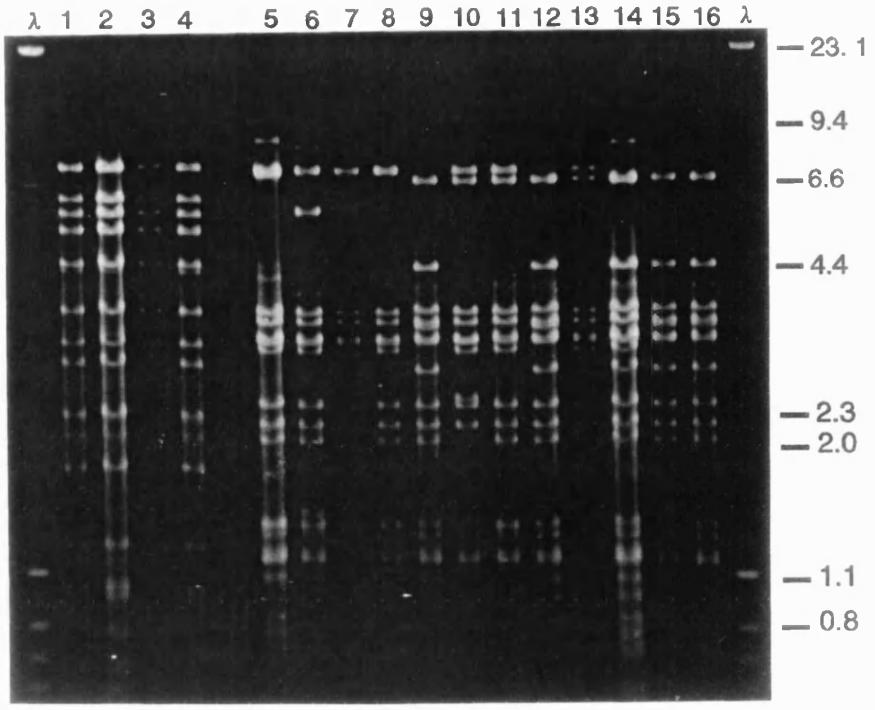


Figure 29 *N. lovaniensis & N. fowleri* EcoR I-Hind III RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hinc III digest. *N. lovaniensis:* (1) HSP 154, (2) Aq/9/1/45D, (3) EX5D/25, (4) C-0490. *N. fowleri*: (5) NHI, (6) MSM, (7) 1518/4, (8) Carter 69, (9) CDC:0487:1, (10) 6088, (11) NF-124, (12) HB-1, (13) NF-59, (14) KUL, (15) NF-3, (16) MCM

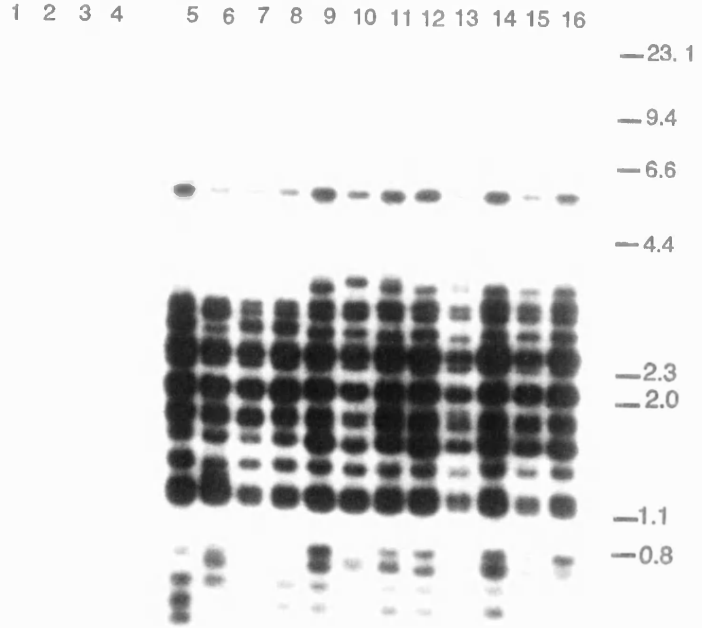


Figure 30 *N. lovaniensis & N. fowleri* EcoR I-Hind III digests probed with pB2.2

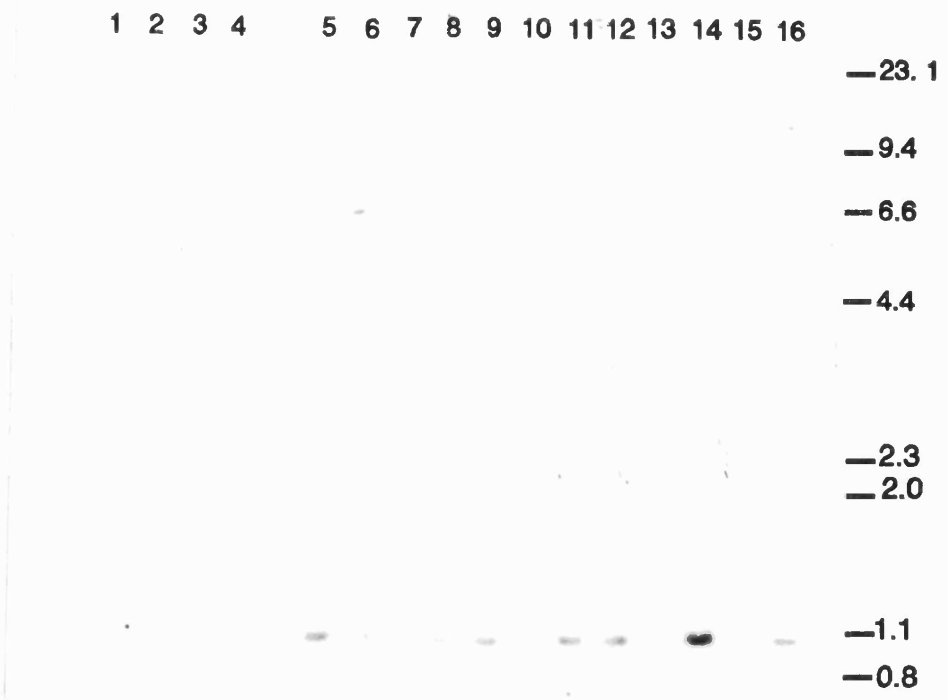


Figure 31 *N. lovaniensis* & *N. fowleri* EcoR I-Hind III digests probed with pB2.3

λ) lambda-Hind III/ΦX-174 RF-Hinc III digest. *N. lovaniensis:* (1) HSP 154, (2) Aq/9/1/45D, (3) EX5D/25, (4) C-0490. *N. fowleri*: (5) NHI, (6) MSM, (7) 1518/4, (8) Carter 69, (9) CDC:0487:1, (10) 6088, (11) NF-124, (12) HB-1, (13) NF-59, (14) KUL, (15) NF-3, (16) MCM

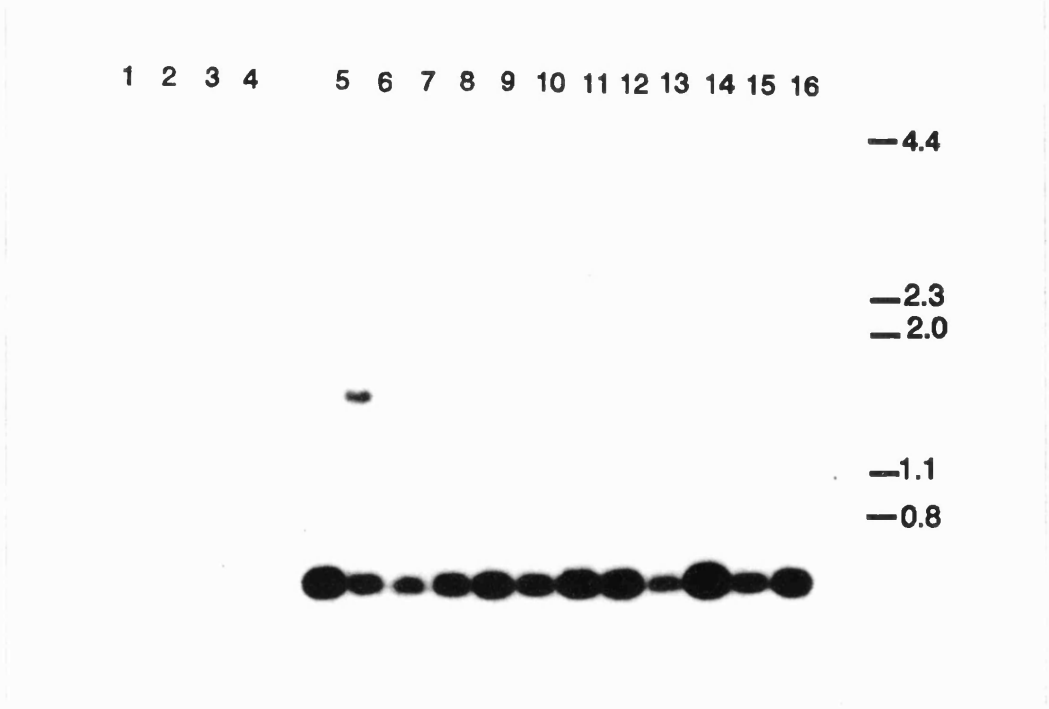


Figure 32 *N. lovaniensis* & *N. fowleri* EcoR I-Hind III digests probed with pB2.2.4

DNA dot-blot hybridisation studies using probes pB2.3 and pB2.2.4 against *Naegleria* spp., other FLA, *T. pyriformis*, *T. vaginalis*, mammalian tissue culture cells, human white blood cells, algae and bacteria is shown in Figure 33. Only hybridisation of the probes to *N. fowleri* whole-cell DNA occurred. No hybridisation was found with *N. fowleri* DNA prepared by the alkaline lysis method which selects for mitochondrial and ribosomal DNA, indicating that the clones are of chromosomal origin.

A weak hybridisation signal was occasionally detected when the probes were tested against *E. coli* JM101 DNA. This was thought to be due to the presence of small amounts of *E. coli* chromosomal DNA that was not completely removed by the alkaline lysis method used to isolate the plasmid clones from the bacterium. This contaminating DNA then becomes labelled with the probe insert and cross-hybridises with *E. coli* DNA. No hybridisation was found with the probes against *N. fowleri* (MCM) DNA extracted by the alkaline lysis method (Figure 33, position A1). This procedure selects for the recovery of double stranded circular DNA such as plasmids and was found to be suitable for the recover mitochondrial DNA and extrachromosomal ribosomal DNA (rDNA) plasmid present in *N. fowleri* (results not shown; Clark *et al*, 1989). This further confirms that the clones are derived from *N. fowleri* chromosomal DNA. Both pB2.3 and pB2.2.4 were found to be of similar sensitivity and could detect as little as 6.25 pg of *N. fowleri* (MCM) DNA if autoradiographs were exposed for up to 5 days (Figure 34). This figure is equivalent to approximately 36 trophozoites assuming a chromosomal DNA content of 0.17 pg/trophozoite (Clark, 1990).

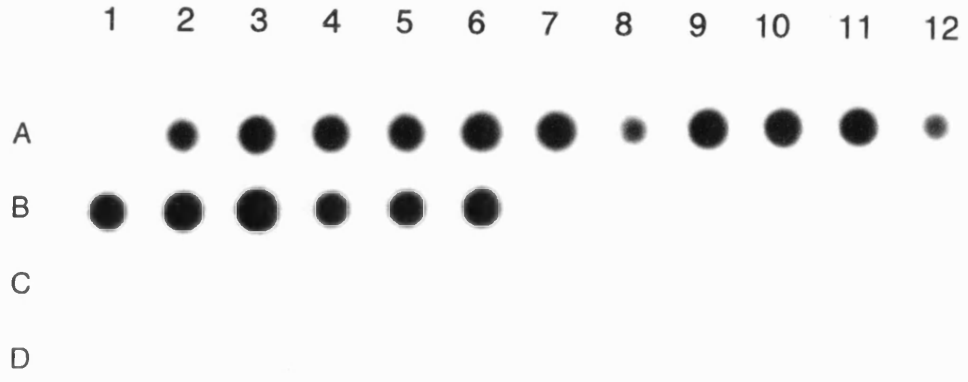


Figure 33 Dot-blots of *N. fowleri* and other organism DNA hybridised with pB2.3

A1 *N. fowleri* (MCM) mtDNA/rDNA; A2-B6 *N. fowleri* strains; B7-B12 *N. lovaniensis* strains; C1-C12 strains of *N. australiensis N. australiensis italica*, *N. andersoni*, *N. andersoni jamiesoni*, *N. jadini*, *W. magna*; D1-D12 strains of *Acanthamoeba* spp., other FLA, *T. pyriformis*, mammalian tissue culture cells, *Trichomonas vaginalis*, algae, *Ps. aeruginosa Legionella* spp. *K. edwardsii* (K10896), *E. coli* (JM101)

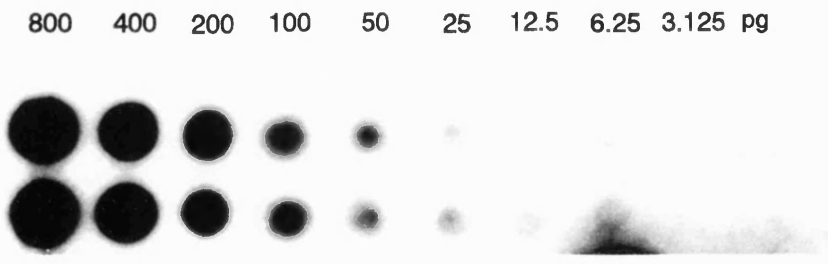


Figure 34 *N. fowleri* (MCM) DNA dilutions hybridised with pB2.3

4.5. Discussion

The genus *Naegleria* and other members of the schizopyrenid amoebae have been shown to carry their the ribosomal RNA genes on extrachromosomal DNA plasmids ([rDNA]: Clark & Cross, 1987; Clark & Cross, 1988a). As rDNA of *N. gruberi* has been shown to cross hybridise with other species of *Naegleria* (Clark *et al*, 1989), this part of the genome was considered to be an unsuitable target for the development of *N. fowleri* specific probes. To minimise the chances of cloning rDNA, the λ phage vector EMBL3 which accepts DNA of size 9-23 kbp and was chosen to construct a genomic library of *N. fowleri* (MCM). This rationale was justified by the isolation of clones which hybridised only to *N. fowleri* chromosomal DNA. Two such clones were subsequently subcloned into the plasmid vector pUC 18 to give the DNA probes pB2.2 and pB2.3, representing a 6.1 and 1.2 kbp inserts respectively. A third probe, pB2.2.4, was derived from pB2.2 and represented an internal 0.8 kbp EcoR I fragment.

Initially, the probes were tested against membrane transfers of restriction endonuclease digests of *N. lovaniensis* and *N. fowleri* whole-cell DNA. As has been previously reported, this permits the detection of RFLPs directly on agarose gel electrophoresis and is a potent technique for the identification of *Naegleria* spp. (De Jonckheere, 1987c). In addition, the RFLPs of *N. fowleri* exhibit interstrain variation that correlates with the geographic origin of isolation. Strains from the Antipodes are distinct from those of Europe, whilst strains which were from the USA are of either profile (De Jonckheere, 1988b; Clark *et al*, 1989; De Jonckheere *et al*, 1992; Pernin & De Jonckheere, 1992). However, here it was found that double digestion with EcoR I and Hind III further differentiated the USA strains of *N. fowleri* of Antipodean profile and thus defines a fourth geographic sub-group of the species. This profile was also found with strain NF-59 from a case of human infection in Czechoslovakia (Kadlec, 1987) and indicates that the USA subgroup also occurs in Europe. Recently, strains of *N. fowleri* from France have been reported that showed RFLPs typical of those from the Antipodes (Pernin & De Jonckheere, 1992). As this profile was only seen in recent isolates of *N. fowleri*, the authors speculated that such strains have only recently emerged in Europe, possibly through the importation from the Antipodes. It would be of interest to examine the French strains for their EcoR I-Hind III RFLPs to establish whether they, like NF-59, are actually related to the USA subgroup defined here.

The origin of the RFLPs detected on agarose gel electrophoresis in this study would appear to be derived from both rDNA and mtDNA. The size of the rDNA plasmid has been estimated as 15.0 kbp in *N. lovaniensis* and 16.5 kbp in *N. fowleri* (Clark *et al*, 1989). Milligan and Band (1988) estimated the size of the mtDNA genome of *N. fowleri* strain HB-1 to be 54.1 kbp by summation of Hind III restriction endonuclease fragment sizes. Here it was found that the sum total of the Hind III RFLPs for *N. lovaniensis* (C-0490) and *N. fowleri* (HB-1) were 67.2 and 68.9 kbp respectively. If the sizes of the rDNA plasmid and mtDNA in *N. fowleri* (HB-1) are combined a total of 70.60 kbp is obtained which approximates to the figure of 68.9 kbp obtained in this study.

The pUC 18 clone pB2.2 was found to contain a repeated element which detected numerous chromosomal RFLPs which showed strain differences also corresponding to their geographic origin. However, this did not permit the additional differentiation of *N. fowleri* strains beyond that of the mtDNA and rDNA RFLPs observed directly on agarose gel electrophoresis. Use of different restriction endonucleases and screening of the EMBL3 λ phage library for the occurrence of other repeated element clones may yet enable the further subtyping of *N. fowleri* strains which may be of value in epidemiological surveys and also help resolve the origins of the geographic dissemination of the organism.

After rigorous screening against DNA from a wide range of *Naegleria* spp. and other eukaryotic and prokaryotic organisms, two clones pB2.3 and pB2.2.4 were found to hybridise only to *N. fowleri* DNA. In the following section the use of these probes in the rapid and specific identification of *N. fowleri* from the environment was investigated.

Table III. Organisms examined in development of *N. fowleri* specific DNA probes

Species	Strain	Origin	Source
N. fowleri	MCM	Bath, England (PAM)	a
	NF-3	Thermal spring water, Bath, England	a
	158-44-3	Power station, Nottingham, England	a
	168-44-5	River water, Nottingham, England	a
	KUL	Belgium (PAM)	b
	NF-59	Czechoslovakia	l
	HB-1	USA (PAM)	b
	6088	USA (PAM)	b
	CDC:0487:1	USA (PAM)	h
	NF-124	Thermal water, USA	b
	Carter 69	Australia (PAM)	b
	CCAP 1518/3 (Morgan)	Australia (PAM)	c
	CCAP 1518/4 (PA-90)	Domestic water supply, Australia	c
	Ng 060	Domestic water supply, Australia	e
	MSM	New Zealand (PAM)	c
	NHI	New Zealand (PAM)	b
N. lovaniensis	Aq/9/1/45D[1]	Aquarium, Belgium	b
	C-0490	Thermal spring water, Bath, England	a
	EX5D/5	Hospital cooling tower, England	a
	HSP154	Hot springs, USA	d
	Ng 045	Water supply, Australia	e
N. australiensis	PP397[1]	Flood water, Australia	c
	4684.11	Thermal spring water, Bath, England	a
	5858.3	Thermal spring water, Bath, England	a
	5858.5	Thermal spring water, Bath, England	a
	LSR34a	Thermal water, France	c
	PV2891	Thermal spring water, Italy	c
	NJ	Pond, India	c
N. australiensis italica	AB-T-F_3[1]	Thermal spa water, Italy	b
N. andersoni	PPMFB-6[1]	Aquarium, Australia	b
N. andersoni jamiesoni	T56E[1]	Tropical fish import, Singapore	b

Table III. Organisms examined in development of *N. fowleri* specific DNA probes

Species	Strain	Origin	Source
N. jadini	CCAP 1518/2[1]	Swimming pool, Belgium	g
N. gruberi	CCAP 1518/1e	Freshwater, USA	g
W. magna	Z503[1]	Bovine faeces, France	b
	NI 13	Pond, India	f
	PAOB CL_4	Pond, Spain	f
A. castellanii	CCAP 1501/1a (Neff)	Soil, USA	g
A. culbertsoni	ATCC 30171 (A-1)	Tissue culture, USA	i
A. lenticulata	BH2	Soil, France	b
A. rhysodes	ATCC 30973	Soil, England	i
A. polyphaga	CCAP 1501/16	Freshwater, USA	g
A. polyphaga	Dav	Keratitis, England	a
A. polyphaga	SHI	Keratitis, England	a
H. vermiformis	9115.1	Bottled mineral water, England	a
	RB-1	Thermal spa water, Bath, England	a
Vannella sp.	BF 1690	Tap water, Northern Ireland	a
Vahlkampfia sp.	B-1270	Bottled mineral water, England	a
Tetrahymena pyriformis	CCAP 1630/1w	Unknown, USA	g
Trichomonas vaginalis	Q442	Vaginal infection	a
	Q7828	Vaginal infection	a
HeLa cell line			a
Hep 2 cell line			a
Monkey kidney cell line			a
Human DNA		White blood cells	a
Scenedesmus quadricande			k
Ulothrix fimbriata			k
Anabaena variabilis			k

Table III. Organisms examined in development of *N. fowleri* specific DNA probes

Species	Strain	Origin	Source
E. coli	JM101		a
K. edwardsii	K10896		a
Ps. aeruginosa	Rb-1760	Thermal spa water, Bath, England	a
	W5789	Contact lens storage case, Bath, England	a
L. pneumophila sg 1	Philadelphia	Legionnaires' disease, USA	j
L. micdadei			j

PAM = from a case of primary amoebic meningoencephalitis

[1] denotes type strain of the species

Strains received from:

[a] S. Kilvington, Public Health Laboratory, Bath, England

[b] Dr J. De Jonckheere, Instituut voor Hygiene en Epidemiologie, Brussels, Belgium

[c] Dr D. Warhurst, London School of Hygiene and Tropical Medicine, London, England

[d] Dr W. O'Dell, Dept of Biology, University of Nebraska at Omaha, USA

[e] Mr P. Christy, State Water Laboratory, Salisbury, South Australia

[f] Dr R. Michel, Ernst-Rodenwald-Institut Medical Parasitologie, Koblenz, Germany

[g] Susan Brown, Culture Collection of Algae and Protozoa, Windermere, Cumbria, England

[h] Dr G. Visvesvara, Centers for Disease Control, Atlanta, Georgia, USA

[i] Dr T. Nerad, American Type Culture Collection, Rockville, Maryland, USA

[j] Dr N. Fry, Central Public Health Laboratory, Colindale, England

[k] Dr J. Wright, School of Biology and Biochemical Sciences, University of Bath, Bath, England

[l] Dr V. Kadlec, Kunz Krajska Hygienicka Stanice, Usti, Czechoslovakia

4.6. PART II: THE DETECTION OF *N. FOWLERI* FROM THE ENVIRONMENT USING DNA PROBES

4.6.1. Summary

The *N. fowleri* specific DNA probes pB2.2.4 and pB2.3 were used to develop a simple and rapid assay for the identification of the organism from environmental samples taken from the thermal springs complex in Bath. Mud samples were inoculated on to non-nutrient agar seeded with *K. edwardsii* (NNA-*K. edwardsii*) and incubated at 44°C. Isolates of thermophilic *Naegleria* were picked into wells of a microtitre plate containing 0.4 N NaOH-10 mM EDTA which caused immediate lysis of the amoebae and inactivated nuclease activity. Each isolate was also inoculated into a corresponding well of a second microtitre plate containing NNA-*K. edwardsii* for incubation and storage. The alkali lysed amoebae were dot-blotted on to nylon membranes and hybridised with the 5'-[α-^{32}P] deoxycytidine triphosphate labelled DNA probe pB2.2.4. A total of 84 separate isolates of thermophilic *Naegleria* were made from the sample site, of which 10 were identified as *N. fowleri* by probe hybridisation. These strains were recovered from the appropriate wells of the NNA-*K. edwardsii* microtitre plate and further investigated by the detection of whole-cell DNA restriction fragment length polymorphisms (RFLPs) which confirmed their identity as *N. fowleri*. Random examination of 5 isolates which did not hybridise with the probes all gave RFLPs typical of *N. lovaniensis*.

4.6.2. Introduction

Following the identification of the natural hot springs in Bath as the source of a fatal case of *N. fowleri* primary amoebic meningoencephalitis in 1978 (Cain *et al*, 1981), regular monitoring of the site has been undertaken for the presence and distribution of the organism (Kilvington *et al*, 1991). These studies have been hampered by the presence of other thermophilic amoebae, particularly *N. lovaniensis*, which predominate in the waters. This necessitates the screening of large numbers of isolates in the hope of identifying *N. fowleri* (Kilvington *et al*, 1991).

As discussed previously (see *1.8. Taxonomy of the Naegleria*), *N. lovaniensis* is nonpathogenic but resembles *N. fowleri* in tolerating growth up to 45°C, cytopathogenicity for tissue culture cell lines and antigenic structure (Stevens *et al*, 1980). When attempting to isolate *N. fowleri* from the environment, samples are usually inoculated on to non-nutrient agar plates seeded with a bacterial food source such as *E. coli* (NNA-*E. coli*) for incubation at 44-45°C to inhibit other less thermotolerant FLA (Griffin, 1972; De Jonckheere, 1978; Warhurst, 1985). However, *N. lovaniensis* grows at a faster rate than *N. fowleri* on NNA-*E. coli* and this may suppress the presence of the latter species.

In this part of the study, the *N. fowleri* DNA probes pB2.3 and pB2.2.4 were investigated for their ability to rapidly identify the organism from samples taken from the thermal springs at the Roman Baths complex in Bath. As was observed in *4.3. Part I: The development of N. fowleri specific DNA probes*, the probes can be contaminated with *E. coli* chromosomal DNA in their preparation from the host strain JM101. This may then result in non-specific hybridisation to the bacterium recovered with FLA from the NNA-*E. coli* medium. To overcome this problem, NNA seeded with *Klebsiella edwardsii* was used for the primary isolation of *Naegleria*. Previous studies have shown the bacterium to be as suitable as *E. coli* for the culture isolation of *Naegleria* and other FLA (S.K. unpublished observation).

4.6.3. Materials and Methods

4.6.3.1. Sample sites. At the Roman Baths complex, the source of the thermal springs rises into the King's Bath from which water overflows to fill a second pool called the Great Bath. Such is the high flow rate from the spring into the King's Bath that water overflows at a sluice gate and is taken down a culvert to eventually discharge into the River Avon. Excess water from the Great Bath flows along a second culvert and meets that from the King's Bath. Four sample points were chosen as indicated in Figure 35: the outlet from the Great Bath (D); the north east corner of the Great Bath (E); the overflow of the Great Bath at the Main Drain from where the water eventually passes into the River Avon (F), and the pool at the base of the Waterfall Point from the King's Bath (G).

4.6.3.2. Isolation of *Naegleria*. Mud samples were collected into sterile polycarbonate containers and transported to the laboratory without delay for processing the same day. The contents were vigorously shaken and the samples spotted on to the surface of square NNA plates (100 mm x 100 mm) seeded with a living suspension of *K. edwardsii* (K10896). For each sample, two lots of sixteen mud or algal spots were distributed evenly over a NNA-*K. edwardsii* plate and allowed to dry into the agar. Plates were incubated at 44 °C in sealed polythene bags and examined daily for up to 5 days for the presence of discrete growths of tightly packed *Naegleria* trophozoites migrating away from the inocula (Figure 36).

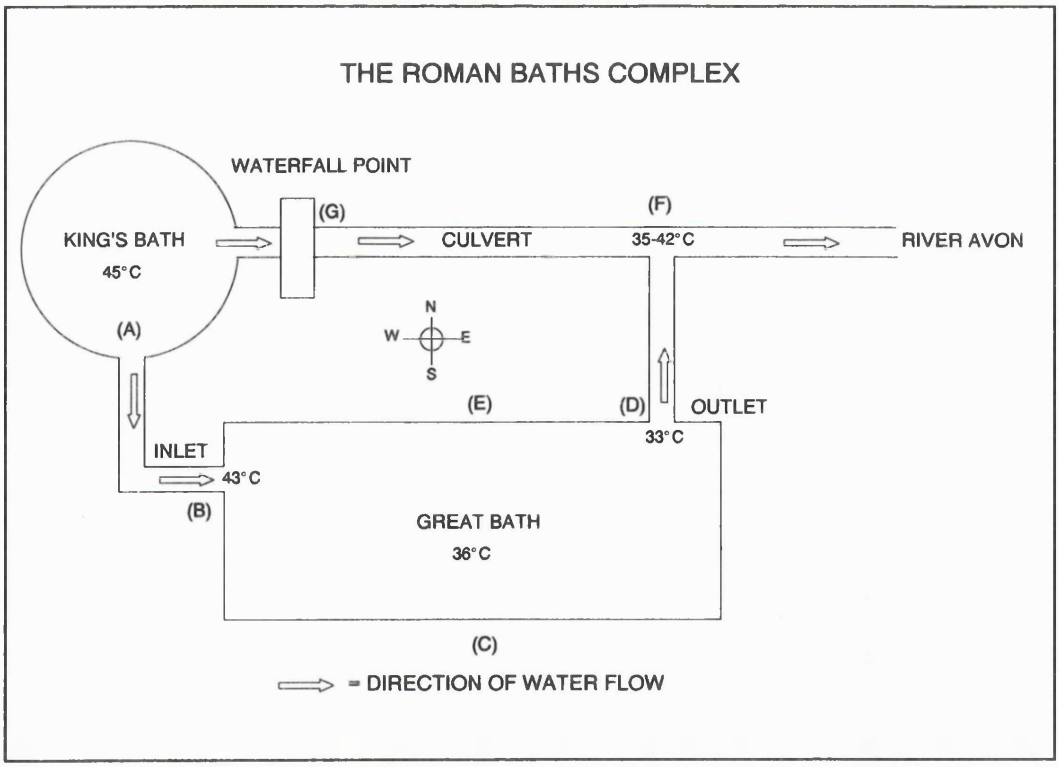


Figure 35 Diagram of the Roman Baths complex and sampling points

Figure 36 *N. fowleri* trophozoites growing on NNA-*K. edwardsii* medium

For each isolate of *Naegleria* on NNA-*K. edwardsii*, a 2-3 cm area of dense trophozoite growth was gently scraped with a disposable 1 $\mu\ell$ bacteriological loop (Nunc, Gibco, Middlesex, England) and inoculated into a well of a 96 place microtitre plate containing 200 $\mu\ell$ of 0.4 N NaOH-10 mM EDTA. Previous studies had shown that about 1 x 10^4 trophozoites were recovered on the loop which is equivalent to approximately 2 ng of *N. fowleri* chromosomal DNA. The 0.4 N NaOH-10 mM EDTA was found to cause the immediate lysis of the trophozoites and inactivation of amoebal nuclease activity. Inoculated plates were sealed with clear adhesive film and stored at 4°C until required for testing.

Using a fresh loop, the primary sampling area was again scraped and inoculated into a corresponding well of a second microtitre plate containing 100 $\mu\ell$ of NNA-*K. edwardsii*. Sufficient trophozoites remain at the primary sampling site to establish growth in the well of the microtitre plate. The plate was then sealed with clear adhesive film and incubated at 37°C for 2 days. Encystment also occurs during incubation and the culture plates may then be stored at 4°C for at least several weeks for subsequent recovery of the strains. The test protocol is outlined in Figure 37.

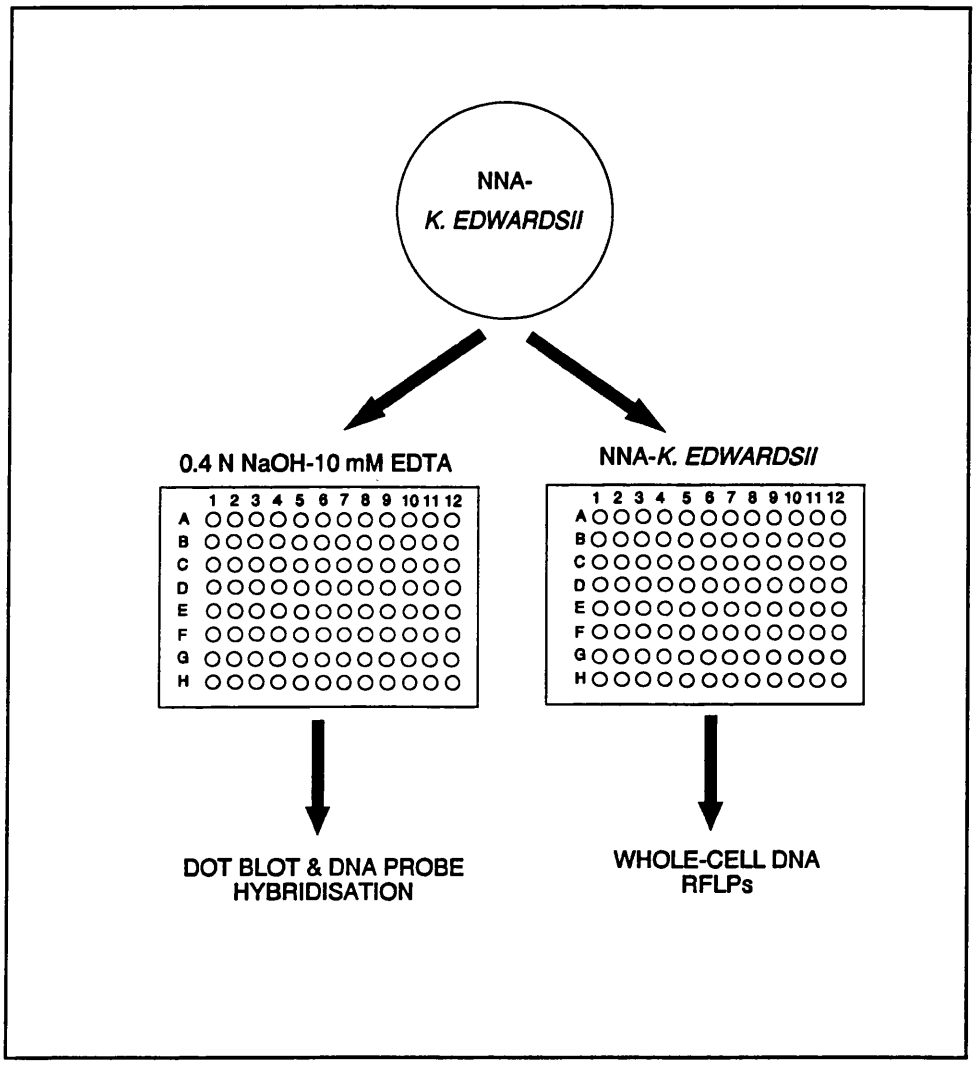


Figure 37 Diagram of DNA dot-blot protocol used to identify *N. fowleri*

4.6.3.3. Detection of *N. fowleri* by DNA probe hybridisation. The primary microtitre plate containing the alkali lysed trophozoites was incubated at 70°C for 15 minutes on the surface of a water bath and chilled on ice. The contents of the wells were then transferred to a Hybond N nylon membrane using a commercial dot-blotting apparatus (Bio-Rad, Hertfordshire, England). This process was facilitated by use of a 12 place multichannel pipette. Controls of *N. fowleri*, *N. lovaniensis* and *K. edwardsii* DNA were also included. After drying at room temperature, the membrane was placed DNA side down on to a transilluminator and exposed for 5 minutes to fix the DNA.

The DNA dot-blots were then hybridised with the *N. fowleri* probe pB2.2.4 labelled with 5'-[α-^{32}P] deoxycytidine triphosphate as described in *4.3. Part I: The development of N. fowleri specific DNA probes* (**Appendix 3.11-3.12**). Following hybridisation, the membranes were washed in 2xSSC-0.1% SDS for 2 x 15 minutes at room temperature, 2xSSC-0.1% SDS for 15 minutes at 65°C, 1xSSC for 30 minutes at 65°C and 0.1xSSC for 30 minutes at 65°C. The membranes were then exposed overnight to X-ray film in autoradiography cassettes containing intensifying screens and developed.

Where positive probe hybridisation was detected on the autoradiographs, the corresponding strain was identified in the appropriate well of the replicate microtitre culture plate and subcultured on to fresh NNA-*K. edwardsii*. The trophozoites were then adapted to growth in #YPNFH (**Appendix 1.3**) and examined for whole-cell DNA RFLPs to confirm their identity as *N. fowleri* (**Appendix 3.2**). Random isolates of *Naegleria* which were negative by probe hybridisation were also tested to confirm their identity as *N. lovaniensis*.

4.6.4. Results

A total of 84 isolates of thermophilic *Naegleria* were made from the four sample sites (Table IV). All the isolates were examined by dot-blot analysis with the *N. fowleri* DNA probe pB2.2.4. and a total of 10 positive strains were identified as shown in Figure 38. Positions A1, B9, D1, H1 and A2, B10, D2, H2 relate to positive and negative controls of known *N. fowleri* and *N. lovaniensis* strains respectively. Reprobing of the membrane with pB2.3 gave identical hybridisation reactions (result not shown).

The outlet from the Great Bath (D) yielded 18 *Naegleria* isolates of which 3 were *N. fowleri*, the north east corner of the Great Bath (E) 20 *Naegleria* and 3 *N. fowleri*, the Main Drain from where the water eventually passes into the River Avon (F) 32 *Naegleria* and 4 *N. fowleri*, and the pool at the base of the Waterfall Point from the King's Bath (G) 14 *Naegleria* but no *N. fowleri* (Table IV).

All isolates giving positive hybridisation with the DNA probe pB2.2.4 were found to have EcoR I RFLPs typical of *N. fowleri* (Figure 39). Examination of 5 isolates which did not hybridise with the probe all gave EcoR I RFLPs typical of *N. lovaniensis* (Figure 39).

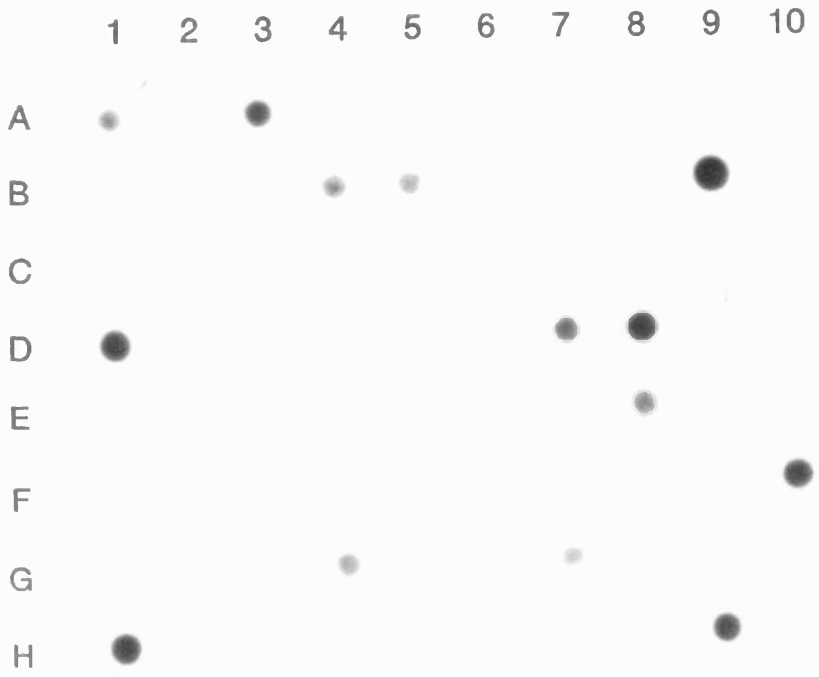


Figure 38 Detection of *N. fowleri* from the Roman Baths by dot-blot hybridisation with pB2.2.4

A1, B9, D1, H1 are positive controls of *N. fowleri*; A2, B10, D2, H2 are negative controls of *N. lovaniensis*

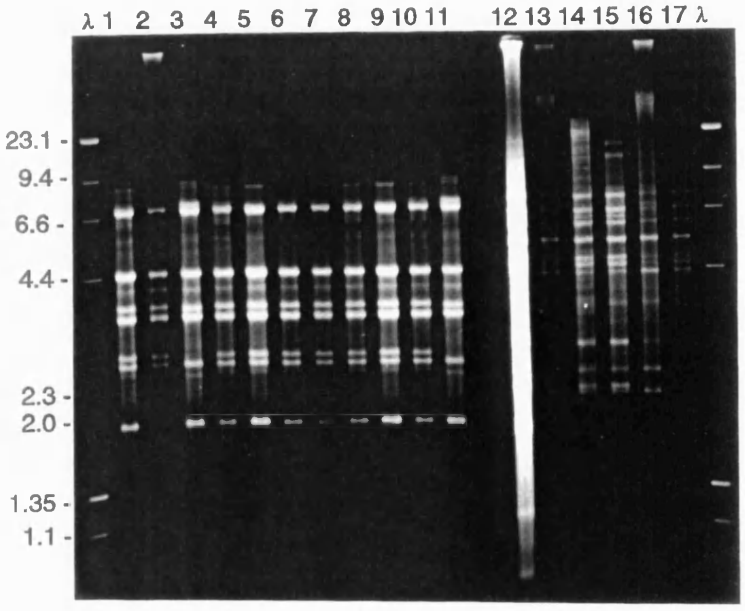


Figure 39 Whole-cell DNA EcoR I RFLPs of Roman Baths isolates of *N. fowleri* and *N. lovaniensis*

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. (1) *N. fowleri* (MCM); (2-11) *N. fowleri* isolates from the Roman Baths identified by probe pB2.2.4.; (12) *N. lovaniensis* (C-0490); (13-17) *Naegleria* isolates from the Roman Baths which did not hybridise with probe pB2.2.4.

Table IV. Isolation of *Naegleria* from the Roman Baths

Sample sites*	Total Nº *Naegleria*	Total Nº *N. lovaniensis***	Total Nº *N. fowleri****
Outlet Great Bath (D)	18	15	3
N.E. Great Bath (E)	20	17	3
Main Drain (F)	32	28	4
Waterfall Point (G)	14	14	0

*Refer to Figure 35

**Identification of *N. lovaniensis* was based on temperature tolerance of 44°C on NNA-*K. edwardsii* and negative hybridisation with the *N. fowleri* specific DNA probes pB2.2.4 or pB2.3. Five random isolates were further identified by characteristic EcoR I RFLPs.

***Identification of *N. fowleri* was by positive hybridisation with probe pB2.2.4 and characteristic EcoR I RFLPs.

4.6.5. Discussion

Using *N. fowleri* specific DNA probes, a simple and rapid assay was developed for the identification of the organism directly following primary isolation. This enabled a total of 10 out of 84 isolates of thermophilic *Naegleria* to be identified as *N. fowleri* from mud samples taken from the thermal springs complex in Bath. All the strains were subsequently confirmed as being *N. fowleri* by their EcoR I whole-cell DNA RFLPs. This represents a significant increase in the usual numbers of *N. fowleri* isolates made from this site and may be due to several reasons. The thermal springs contain large numbers of thermophilic *Naegleria* of which *N. lovaniensis* is by far the predominant species (Kilvington *et al*, 1991). In previous surveys, only a small proportion of isolates were examined further for species identification because of the practical constraints of the isoenzyme or whole-cell DNA analytical methods available. It was also noted that *N. lovaniensis* grows at a faster rate than *N. fowleri* on NNA-*E. coli* or NNA-*K. edwardsii* media and thus may suppress the presence of the latter during primary culture isolation. Accordingly, the DNA probe strategy developed here for the identification of *N. fowleri* enables all isolates to be readily sampled as soon as they emerge from the inocula on the culture plates and before possible overgrowth by *N. lovaniensis* can occur.

It is essential that the target DNA be single stranded when immobilised on to nylon membranes for subsequent probe hybridisation. This is usually achieved by alkali or heat denaturation of the DNA (Reed & Mann, 1985; Sambrook *et al*, 1989). Here, it was found that inoculation of amoebae into 0.4 N NaOH-10 mM EDTA resulted in the immediate lysis of the trophozoites, with inactivation of nuclease activity. This led to the development of a simple sampling strategy that, in conjunction with the use of microtitre plates and a commercial DNA dot-blotting apparatus, readily enabled *N. fowleri* to be identified among large numbers of other thermophilic *Naegleria* isolates soon after primary culture isolation.

Sparagano (1993a & 1993b) has described the detection of *N. fowleri* by the PCR in conjunction with a DNA probe derived from an internal region to the amplified product. In artificially seeded water samples, the presence of environmental sediment was inhibitory to the PCR and failed to detect *N. fowleri*. Only when amoebae were first isolated by culture on NNA-*E. coli* medium was the PCR and probe hybridization able to detect *N. fowleri*. Whilst the PCR is a highly sensitive technique, capable of detecting as little as one *N. fowleri* cell (Sparagano, 1993a), the protocol described is complicated and time consuming. Following culture isolation, DNA must be extracted from the amoebae, PCR performed, the amplified product separated by agarose gel electrophoresis and then membrane transferred for probe hybridization. In the DNA probe detection of *N. fowleri* described here, amoebae are tested directly from primary isolation plates and no separate DNA extraction step is involved. Following the primary isolation of thermophilic *Naegleria* the process of dot-blotting, probe hybridisation and autoradiography is complete within 2 days. This represents a considerable advantage over conventional identification methods such as isoenzyme and whole-cell DNA RFLP analysis which usually require strains to be adapted to axenic culture and do not lend themselves readily to the examination of large numbers of isolates which are frequently encountered in natural environments (Kilvington *et al*, 1991).

A disadvantage of the described method may be considered from the use of 5'-[α-^{32}P] deoxycytidine triphosphate to label the DNA probes. Besides the potential hazards associated with the use of radio-nucleotides, the half-life of the isotope is 14 days and probes must be prepared fresh each time. Several nonradioactive methods for the labelling of DNA are now available commercially, most commonly using a biotin or digoxigenin molecule coupled to a nucleotide in the DNA labelling reaction (Wolcott, 1992). The detection of probe hybridisation is accomplished by incubation with an enzyme labelled antibody specific to the modified nucleotide and visualisation by colorimetric or chemiluminescent assay (Wolcott, 1992). Typically, probes labelled by such methods are stable for many months, can be reused several times and the detection of hybridisation is usually complete within hours rather than days. Although not extensively investigated, digoxigenin labelling of probe pB2.3 in conjunction with chemiluminescent detection has been tested against dot-blots of *N. fowleri* and *N. lovaniensis* DNA. The results were, however, disappointing with nonspecific hybridisation occurring. The use of more stringent hybridisation and washing conditions may resolve this problem and lead to the development of nonradioactive DNA probes for the detection of *N. fowleri* with noted advantages.

5. THE DEVELOPMENT OF THE POLYMERASE CHAIN REACTION FOR THE IDENTIFICATION OF *NAEGLERIA FOWLERI*

5.1. Summary

The *N. fowleri* specific DNA probe pB2.3 was partially sequenced and oligonucleotide primers synthesised to the 5' and 3' ends for use in a polymerase chain reaction (PCR) assay for the organism. After rigorous evaluation PCR was shown to amplify only *N. fowleri* DNA. In sensitivity studies, down to 1 pg of purified DNA could be detected on agarose gel electrophoresis following 35 PCR amplification cycles or 100 fg in combination with probe hybridisation. This is equivalent to approximately 6 and 1 trophozoite respectively. A simple method for the rapid isolation of DNA from *N. fowleri* trophozoites or cysts was developed based on incubation of amoebae in Taq polymerase buffer with proteinase K. Following boiling to inactivate the proteinase K, addition of primers, nucleotides and Taq polymerase, PCR could be performed directly on the sample. By this method, 10 *N. fowleri* trophozoites or cysts could be detected after 35 PCR cycles or as little as 1 organism after 45 cycles. In conjunction with the rapid DNA isolation method, the PCR was used to identify *N. fowleri* directly from primary isolation cultures made from the thermal springs complex in Bath.

5.2. Introduction

The polymerase chain reaction (PCR) is an *in vitro* amplification procedure that can copy a specific segment of DNA by as much as 10^8-fold. Since the technique was first described in 1985 (Saiki *et al*, 1985) the specificity, sensitivity and versatility of the PCR has revolutionised molecular biology and represents one of the most substantial technical advances in this field in the past decade. The PCR involves three stages, heat denaturation of the target DNA, annealing of oligonucleotide primers, and amplification of the DNA by the enzyme Taq polymerase. First, the double stranded DNA is heated (94°C-96°C) to break the hydrogen bonds and render it single stranded. This is required to enable the primers to anneal to the DNA. Here, two oligonucleotide primers, usually 20-30 bp in length, attach to complementary sequences on opposite strands of the DNA template. The primers are chosen to encompass the desired region of DNA that is to be amplified. Annealing of the primers is performed at lower temperatures (usually between 50°C-60°C) and since they are present in excess to the DNA template, this favours the formation of the primer-template complex rather than the reassociation of the DNA strands. The third step is the actual synthesis of a complementary second strand of new DNA. This occurs through the extension of each annealed primer by Taq polymerase at 72°C in the presence of excess deoxyribonucleotide triphosphates. The enzyme copies the DNA starting from the 3' end of each primer resulting in the synthesis of DNA in a 5' to 3' direction. The process of heat denaturation, primer annealing and Taq polymerase amplification is then repeated in a successive round of cycles. Since all previously synthesised

products act themselves as templates for new primer-amplification reactions, the result is a geometric amplification of new DNA products to levels detectable by gel electrophoresis.

In the initial development of the PCR, the Klenow fragment of *Escherichia coli* DNA polymerase I was used for amplification (Saiki *et al*, 1985). As the Klenow polymerase is heat-labile it meant that fresh enzyme had to be added after each cycle. The use of thermostable DNA polymerase purified from the bacterium *Thermus aquaticus* (Taq) has greatly simplified the procedure. The enzyme is stable at the temperatures needed to denature the double stranded DNA and has optimal activity at 72°C (Gelfand, 1989). This also enables annealing to be done at higher temperatures and avoids the nonspecific attachment of primers and subsequent amplification of undesired DNA that can occur with the use of Klenow polymerase.

Although the PCR can be performed with a single short oligonucleotide primer to produce random amplification of DNA, conventional PCR requires the use of two primers that will attach to complementary sequences on opposite strands of the DNA template. The choice of primers is made from knowledge of the nucleotide sequence in the region of DNA to be amplified. The process of determining the nucleotide sequence is made by either using the chemical cleavage method developed by Maxam and Gilbert (1977) or the chain termination method developed by Sanger and colleagues (1977).

In the Sanger DNA-sequencing procedure, which was used here, 2',3'- dideoxynucleotides (ddNTPs) of each of the four bases are prepared. These molecules can be incorporated into DNA by *E.coli* DNA polymerase because they have normal 5' triphosphate. However, once incorporated into a growing DNA strand they cannot form a phosphodiester bond with the next incoming dNTP. Growth of that particular DNA chain is thus halted. The Sanger sequencing reaction consists of a DNA strand to be sequenced, a short piece of labelled DNA (the primer) that is complementary to the end of that strand, a carefully controlled ratio of a particular dideoxynucleotide with its normal deoxynucleotide, and the other three dNTPs. When DNA polymerase is added, normal polymerization will begin from the primer; when a ddNTP is incorporated, the growth of that chain will stop. If the correct ratio of ddNTP:dNTP is chosen, a series of labelled strands will result, the lengths of which are dependent on the location of a particular base relative to the end of the DNA.

With both sequencing methods, the sets of labelled fragments obtained from each of the four reactions are run side by side on a polyacrylamide gel which separates DNA fragments according to size. Following autoradiography of the gel, the pattern of bands on the X-ray film is read to determine the sequence of the DNA.

Once the sequence of a DNA segment is obtained, complementary primers can then be chemically synthesised. The process is based on the ability to protect specifically the chemical reaction occurring at either the 5' or the 3' end of a mono- or oligonucleotide. This is done by attaching a large blocking group

onto either the 5' or the 3' hydroxyl. Different blocking groups are used, which can be removed by treatment with an acid or base. Thus a 5' blocked mononucleotide can be chemically condensed with a 3' -blocked molecule, resulting in a dinucleotide blocked at both ends. The 5' or the 3' blocking group is then removed by either acid or base exposure and the dinucleotide reacted with an appropriately unblocked mono- or dinucleotide. This cycle of condensation, removal of one or the other blocking group, and recondensation is repeated many times until the oligonucleotide of the desire sequence length is obtained. The process is now performed using automated synthesizer machines where the first nucleotide to be made is attached to a solid support. Subsequent nucleotides are attached consecutively, with washing of the support matrix between each step.

The sensitivity, specificity and versatility of the PCR has found application in most fields of medical and biological science. Examination of the *Journal of Clinical Microbiology* **31**: (10-11) showed that approximately one third of the published articles used PCR in the research. PCR using primers that amplify species specific DNA have been widely used in the diagnosis of infectious diseases. Here, the PCR is of great value when identifying organisms that grow slowly or not at all *in vitro*, for example: *Mycobacterium tuberculosis* (De Wit *et al*, 1990), *M. leprae* (Klatser *et al*, 1993), *Legionella* (Stanbach, *et al*, 1989; Mahbubani *et al*, 1990; Bej *et al*, 1991a), and many viruses including those responsible for human immunodeficiency and hepatitis (Rogers, *et al*, 1989). The PCR for the detection of human parasitic protozoa has also been widely reported. Examples including *Toxoplasma gondii* (Burg *et al*, 1989), *Giardia* (Mahbubani *et al*, 1991 & 1992), *Trypanosoma cruzi* (Moser *et al*, 1989), *Trichomonas vaginalis* (Riley *et al*, 1992), and *Entamoeba histolytica* (Tachibana *et al*, 1991).

PCR typing of strains is also possible using a single short primer, usually about 10 bp in length. The complementary sequence for primers of this length can be predicted to occur frequently in the genome. PCR amplification occurs whenever two correctly orientated copies are close enough for the PCR to operate efficiently (Welsh & McClelland, 1990; Williams *et al*, 1990; Welsh *et al*, 1991a). This random amplification of polymorphic DNA (RAPD) has been used to generate 'fingerprints' in a wide range of higher eukaryotic organisms (Welsh & McClelland, 1990; Williams *et al*, 1990; Riley *et al*, 1991; Welsh *et al*, 1991a; Riedy *et al*, 1992), fungi (Niesters *et al*, 1993; Van Belkum *et al*, 1993a), bacteria (Akopyanz *et al*, 1992; Mazurier & Wernars, 1992; Jordens *et al*, 1993). RAPD typing of the protozoa *Giardia duodenalis* and *Schistosoma* spp. has also been described (Homan *et al*, 1992; Barral *et al*, 1993; Van Belkum *et al*, 1993b).

Sequencing of DNA with Taq polymerase in the PCR is also possible by the Sanger chain termination method (Gyllensten, 1989). This approach has the advantage that the chain elongation is accomplished at elevated temperatures that can overcome unreadable regions of nucleotide "compression" often seen when sequencing with Klenow polymerase (Gyllensten, 1989). The PCR can also be used in the sequencing of DNA previously amplified using universal primers that flank the ribosomal RNA (rRNA) genes (Embley, 1991). Sequence analysis of rRNA genes have been widely used for inferring phylogenetic relationships

between bacterial and eukaryotic species, including amoebae, due to their important functional role (Baverstock *et al*, 1989; Lynn & Sogin, 1988; Mindell & Honeycutt, 1990; Embley, 1991; Fry *et al*, 1991; Olsen *et al*, 1992). While rRNA genes are informative at the species and higher levels, they are often too highly conserved to reveal differences within species. However, the internal transcribed spacers (ITS) in eukaryotic organisms between the 18S, 5.8S and 28S genes, though they are transcribed, do not form part of the mature rRNAs. The ITS being virtually free from functional constraints are therefore free to evolve at a faster rate. Consequently sequences have been successfully used to explore intraspecific relationships (Bakker, 1992). DNA sequencing is, however, expensive and time consuming and does not lend itself to the examination of numerous strains. By the PCR, it is possible to amplify regions of rRNA genes and ITS and digest the product with restriction endonuclease digestion to reveal restriction fragment length polymorphisms (RFLPs). This 'riboprinting' approach has been used successfully in the differentiation of several protozoa including *Entamoeba* (Clark & Diamond, 1991a & 1991b) and *Tetrahymena* (Orias *et al*, 1991).

The PCR has been variously applied to free-living amoebae. Amplification of repetitive DNA has been described for identifying *N. fowleri* from purified nucleic acids and in crude preparations of infected mouse brains (McLaughlin *et al*, 1991) and also from environmental culture isolates (Sparagano, 1993a & 1993b). For *Acanthamoeba*, sequence data of cloned mitochondrial DNA from *A. polyphaga* and the 18S and 5.8S ribosomal RNA genes of *A. castellanii* have been used to construct primers for the differentiation of the genus, species and strains (Vodkin *et al*, 1992). RAPD typing has been used to differentiate *Naegleria* spp. and was also found to detect minor variations in three out of twenty *N. fowleri* strains (Van Belkum *et al*, 1992). Amplification of the small subunit ribosomal DNA of *Naegleria* using universal eukaryotic primers for this region (Embley, 1991) has demonstrated the presence of a Group I intron in *N. andersoni*, *N. andersoni jamiesoni*, *N. australiensis italica* and some strains of *N. gruberi* but not *N. jadini*, *N. australiensis*, *N. fowleri* or *N. lovaniensis* (Embley *et al*, 1992; De Jonckheere, 1993). This approach has also been used to amplify the 16S-like rRNA coding region for sequence analysis in the phylogenetic analysis of *N. gruberi* and other vahlkampfiid amoebae (Clark & Cross, 1988b; Hinkle & Sogin, 1993).

Potentially, the PCR is a highly specific, sensitive and rapid method for the identification of microbes. Accordingly, the value of the technique for the rapid identification of *N. fowleri* using primers derived from the species specific probes pB2.3 and pB2.2.4 described in *4.3. Part I: the development of N. fowleri specific DNA probes* was investigated.

5.3. PART I. DNA SEQUENCING OF *N. FOWLERI* pUC 18 CLONES pB2.3 AND pB2.2.4

5.3.1. Materials and Methods

5.3.1.1. Preparation of DNA clones. Methods for the isolation and purification of the pUC 18 plasmid clones pB2.3 and pB2.2.4 from *E. coli* (JM101) are described in **Appendix 3.18**. Briefly, transformed *E. coli* was grown in Terrific Broth (**Appendix 1.12**) at 37°C overnight with vigourous shaking. Plasmid DNA was isolated by the alkaline lysis method but was also further purified by silica absorption (**Appendix 3.15**). DNA concentrations were estimated by comparison with standards spotted on to agarose plates containing the fluorochrome dye Hoechst H33258 and visualisation by UV illumination (Rieber & Rieber, 1990) as described in **Appendix 3.5**.

5.3.1.2. Sequencing of DNA clones. The Sequenase® Version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio, USA) was used for sequencing double stranded DNA following the manufacturer's protocol. The precise details of the methods used for the sequencing of the plasmid clones are detailed in **Appendix 3.21**. Briefly, plasmid DNA was denatured (rendered single stranded) by incubation in 0.2 N NaOH at room temperature for 5 minutes. The single stranded DNA (ssDNA) was then passed down a Sephadex G-50 column equilibrated with TE (7.5) buffer to remove the NaOH and used immediately for sequencing (**Appendix 3.21.1-3.21.2**). Each clone was sequenced from both the 3' and 5' ends using primers designed to anneal to the appropriate ends of the multiple cloning site of the pUC 18 plasmid. For each reaction, an annealing mix (10 $\mu\ell$) was prepared with 3 μg of ssDNA template in 7 $\mu\ell$ of TE buffer (see above), 1 $\mu\ell$ of 2 μM primer and 2 $\mu\ell$ of x 5 reaction buffer (200 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl). The mix was heated at 65°C for 2 minutes and cooled slowly to room temperature. The labelling reaction contained the annealing mix, 0.5 $\mu\ell$ of adenosine 5'(α-^{35}S)thiotriphosphate (3000 Ci/mmol, Du Pont), 1 $\mu\ell$ of dithiothreitol (DTT: 0.1 M) and 2 $\mu\ell$ dNTP (0.2 μM each of dGTP, dCTP and dTTP). Sequenase® 2.0 (13 U/$\mu\ell$ was diluted 1:8 in cold buffer (10 mM Tris-HCl, pH 7.5; 5 mM DTT; 0.5 mg/ml BSA); 2 $\mu\ell$ of diluted enzyme was added last; incubation was at 20°C for 3 minutes. Labelling mix (3.5 $\mu\ell$) was added to the ddG, ddA, ddT and ddC termination mixes (2.5 $\mu\ell$ each). The termination mix contained ddGTP, ddATP, ddTTP or ddCTP (8 μM) and 4 deoxynucleotides (80 μM each). After incubation at 37°C for 5 minutes, 4 $\mu\ell$ of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added.

Following heating at 80°C for 2 minutes, the sequencing reactions were electrophoresed on 6% polyacrylamide gels (0.4 mm thick x 38 cm long) in TBE buffer at 40 mA for 1.5 h or 4.5 h (**Appendix 3.22**). Gels were fixed in 10% acetic acid-10% methanol for 30 minutes, dried on to the glass plate at 80°C for 30 minutes and exposed overnight to Fuji RX100 film for autoradiography.

5.3.1.3.Synthesis of oligonucleotide primers. The nucleotide sequence of the 5, and 3' ends of each DNA clone was read and used to make forward and reverse primers on an Applied Biosystems oligonucleotide synthesizer by the School of Biology and Biochemistry, University of Bath, Bath, England. Oligonucleotides were purified from the synthesizer columns by elution with concentrated ammonia solution and incubation at 55°C, followed by ethanol precipitation (**Appendix 3.23**). The primers were dissolved in ddH_2O and the purity and concentration of the oligonucleotides was measured by spectrophotometric analysis between 300 nm and 240 nm. Primers were aliquoted and stored at -20°C.

5.4.PART II. DEVELOPMENT OF THE PCR FOR *N. FOWLERI*

Such is the sensitivity of the PCR that cross-contamination leading to false positive results can be a serious problem. To minimise this event the following procedures, as recommended by Kwok & Higuchi (1989) were employed:

i. Preparation of DNA samples, PCR reactions and the thermal cycling were done in separate rooms. Inoculation of reaction tubes with the PCR components and template DNA was made under an operating laminar flow cabinet.

ii. Latex gloves were worn throughout and changed regularly.

iii. Automatic pipettes, designated only for PCR, were used with autoclaved sterile pipette tips and reaction tubes. Pipette tips plugged with a filter material (ART, Northumbria Biologicals Ltd, Northumbria, England) were used to dispense the DNA solutions. These tips are designed to prevent aerosol contamination of the pipette barrel during manipulation of the DNA.

iv. The working solution of PCR components (Taq polymerase buffer, dNTPs and primers) without Taq polymerase was exposed to UV light for 5 minutes using a transilluminator covered with a fresh sheet of Saran® Wrap. This degrades double stranded DNA that may be contaminating the preparation but does not affect the single stranded primers (Sarkar & Sommer, 1990).

v. A negative control tube containing no DNA template was included in every set of PCR tests.

5.4.1.Materials and Methods

5.4.1.1.Organisms studied and isolation of DNA. The list of organisms studied and methods for the isolation of DNA from the strains is as described in *4.3. Part I: the development of N. fowleri specific DNA probes.*

5.4.1.2. The PCR. Taq polymerase and enzyme buffer was obtained commercially (Promega, Southampton, England) and stored at -20°C. The X10 enzyme buffer comprised: 500 mM KCl; 100 mM Tris-HCl, pH 9.0 at 25°C; 15 mM $MgCl_2$ and 1.0% Triton X-100. Ultrapure dNTPs were supplied by Pharmacia LKB Ltd, Milton Keynes, England and diluted to 10 mM each with dH_2O. Primers were adjusted to 10 μM each in dH_2O. Each PCR was performed in a final volume of 100 $\mu\ell$ in a 0.5 ml microcentrifuge tube and comprised: X1 Taq polymerase buffer; 0.2 mM of each dNTP; 1 μM of each primer; 2.5 U of Taq polymerase; and ~100 ng of DNA. The reaction mix was prepared in 80 $\mu\ell$ of dH_2O and added to 20 $\mu\ell$ of DNA. Two drops of sterile mineral oil were added to the tube from a 1 ml disposable plastic pasteur pipette. After a centrifuging at 12,000 rpm for 3 seconds the tubes were subjected to the thermal cycling steps of the PCR.

As several PCR reactions were usually performed at a time, a stock working solution ("Master Mix") was prepared comprising of Taq polymerase, enzyme buffer, dNTPs and primers so that 80 $\mu\ell$ aliquots could be added to the appropriate number of DNA samples each contained in 20 $\mu\ell$ of dH_2O (**Appendix 3.24**). The PCR was performed in a Perkin-Elmer thermal cycler machine. For primers complementary to the pB2.3 clone, the temperature programme consisted of 1 cycle with 5 minutes at 95°C, 1 minute at 55°C and 1.5 minutes at 72°C; 33 cycles of 1 minute at 95°C, 1 minute at 55°C and 1.5 minutes at 72°C; and finally 1 cycle at 1 minute at 95°C, 1 minute at 55°C and 7 minutes at 72°C.

5.4.1.3. Analysis of PCR products. The oil on top of the reactions can interfere with subsequent analysis of the PCR product. The tubes were therefore placed at -70°C for 10 minutes and the oil removed with a pipette from the frozen PCR mixture below. Aliquots of 20 $\mu\ell$ from each reaction were analyzed on a 1.2% agarose-TBE gel at 4 v/cm for 2 hours (**Appendix 3.4**). Ethidium bromide at 0.5 μg/ml was included in the agarose gel and electrophoresis buffer.

Following electrophoresis, the gels were photographed under shortwave UV transillumination using Polaroid 665 film and a Kodak Wratten #23A orange filter. The gels were then alkaline blotted (**Appendix 3.19**) on to Hybond N nylon membranes (Amersham, Buckinghamshire, England). Membranes were hybridised with the pB2.3 DNA clone labelled with 5'-[α-^{32}P] deoxycytidine triphosphate as described in **Appendix 3.11**. PCR and hybridisation studies was also performed with ten-fold dilutions of *N. fowleri* (MCM) DNA from 10 ng to 100 fg.

The PCR product from the pB2.3 primers was also digested with the restriction endonucleases Alu I and Hae III (Northumbria Biologicals, Northumbria, England). Following PCR, 18 $\mu\ell$ aliquots were removed and 2 $\mu\ell$ of X10 restriction enzyme buffer and 1 $\mu\ell$ of enzyme (6 U and 8 U respectively) added. After incubation at 37°C for 3 hours, the samples were analyzed on 1.2% agarose TBE gels. The activity of these enzymes was not affected by the components of the PCR as identical results were obtained if the amplification product was first purified by passing through a Sephadex G-50 column equilibrated with dH_2O before restriction endonuclease digestion (**Appendix 3.21.1**).

5.4.2. Results

The partial DNA sequence of clones pB2.3 and pB2.2.4 obtained from near the ends of the multiple cloning site of the pUC 18 plasmid is given below. The position of guanine nucleotides is shown in lower case (g) to avoid confusion with those of cytosine (C). The region of the sequences used to synthesize the oligonucleotide primers are underlined.

pB2.3 forward (5') sequence

5' TCgAgAAAgTgTCATTACTTTgCAAgCgAAgATgAgACACTATTTgTgTTCCAAAgCTTTgAATg
CAAgTAAgAAAgCTATCgAATggATTCAAgCAAAAAgTAAgAgTTCATTAACATACAAACAA
TCACAACAATCCCTCCAAAgAATCCTCACTCTgCAAgCTCTgATgAgATCCTTCCACgAg

The pB2.3 forward primer (p3f-gCTATCgAATggATTCAAgC) is a 20-mer comprising 6 adenine, 4 cytosine, 5 guanine and 5 thymine nucleotides. The G+C content is 45% and the TmS (4 [G+C] + 2 [A+T]) is 58°C.

pB2.3 reverse (3') sequence

5' TCAAAACAgATgTAATggATTgTgATTTTTTgTggAAATgTTgTgAAgAAgATTCCATCAAT
AgggATTgAACACTACTCgTggAAggCTTATTgAAATgATCATCTTgTgTTTCTTTCTTTTg
TAAgAAgAgTgTTgAATCTACAgATTCgAATATTggAggAgCAgTCgATgATTgg

The pB2.3 reverse primer (p3r-CACTACTCgTggAAggCTTA) is a 20-mer comprising 5 adenine, 5 cytosine, 5 guanine and 5 thymine nucleotides. The G+C content is 50% and the TmS is 60°C.

pB2.2.4 forward (5') sequence

5' gAgCTCggTACCCgATCTCTAgAgTCgCAATggTAAggCgAggAATgACAACCCAAgAATCAAT
ACTgCTgACTTTgAAAAgggTCTTgCTACTgATgTTCAATCTT

The pB2.2.4 forward primer (p4f-gAgCTCggTACCCgATCTCT) is a 20-mer comprising 3 adenine, 7 cytosine, 5 guanine and 5 thymine nucleotides. The G+C content is 60% and the TmS is 64°C.

pB2.2.4 reverse (3') sequence

5' TCAAACTACATAATACCgTTTACACTTggggTggATTAACAAgTAATggAATTATCAATgAgTT
gATTACATTggAATTggACAAAATgAAACTTTCCCCTAAAAgACAggACAACAAAATTTCTg
AAAggATATTTCATAgTTTTgATTTTgTTgTTCACTCAATgTATTTgTgATTATTggAggTATCgAC

The pB2.2.4 reverse primer (p4r-ACCgTTTACACTTggggTggA) is a 21-mer comprising 4 adenine, 4 cytosine, 7 guanine and 6 thymine nucleotides. The G+C content is 52% and the TmS is 64°C.

A region of the autoradiograph used to read part of the forward and reverse sequence for both clones is shown in Figure 40.

The results of PCR with the primer set p3f and p3r using purified DNA of *Naegleria* spp., other FLA, protozoa, algae and bacteria is shown in Figure 41. A single 1.5 kbp product was detected in all the strains of *N. fowleri* examined. No amplification products were obtained with any of the other organism DNA studied. Under lower stringency conditions, in which the PCR annealing was done at 45°C, additional smaller products were formed with *N. fowleri* DNA but not with *N. lovaniensis* (results not shown). Following alkaline transfer of the gel and hybridisation with the DNA probe pB2.3 only the 1.5 kbp *N. fowleri* PCR product was detected (Figure 39).

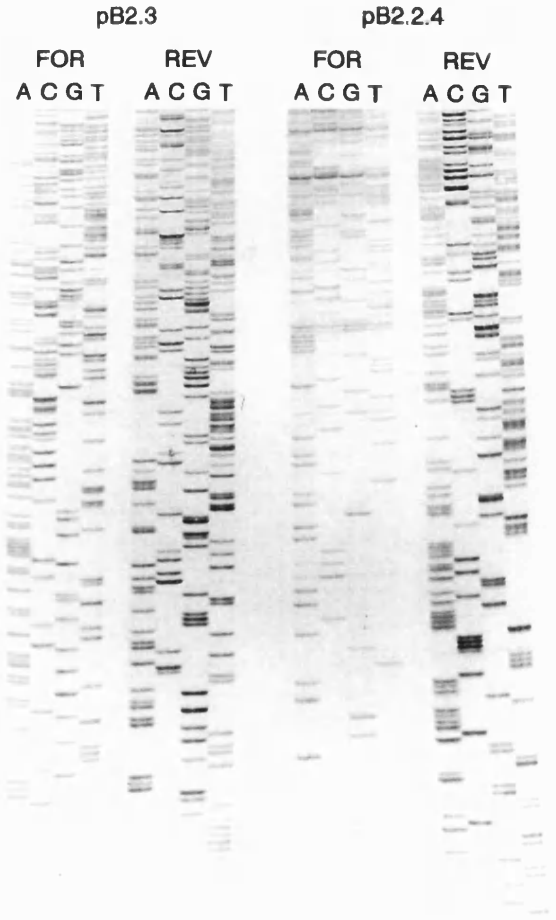


Figure 40 Autoradiograph showing the partial nucleotide sequence of the *N. fowleri* DNA probes pB2.3 & pB2.2.4

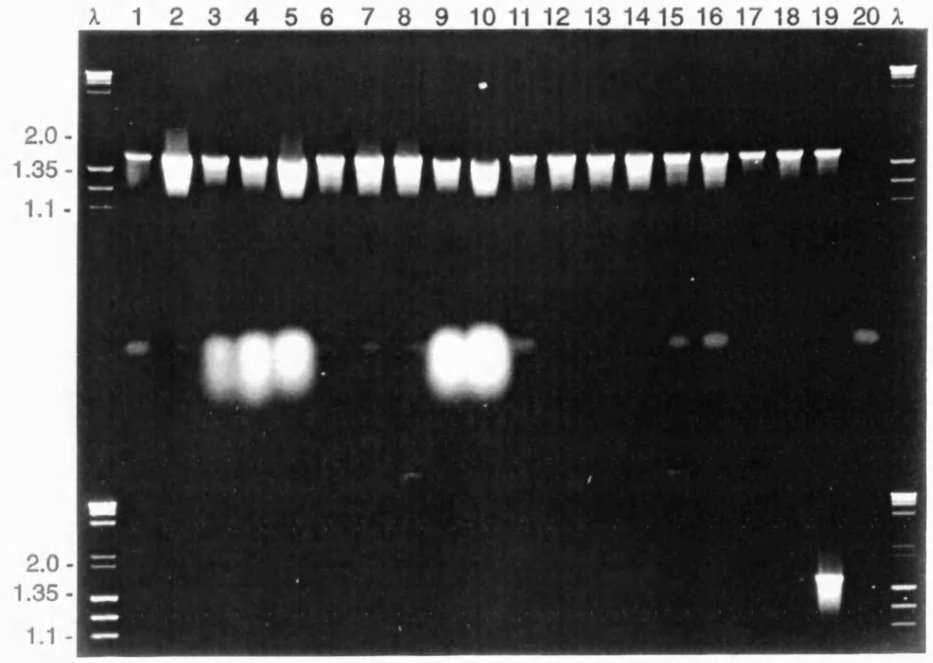


Figure 41 Agarose gel showing the PCR amplification of *N. fowleri* DNA only using primer sets pB2.3

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. (1-19) *N. fowleri* strains; (20) negative control. Lower lanes (1A-18A) strains of *N. lovaniensis*, *N. australiensis N. australiensis italica*, *N. andersoni*, *N. andersoni jamiesoni*, *N. jadini*, *W. magna*, *Acanthamoeba* spp., *T. pyriformis*, *T. vaginalis*, mammalian tissue culture cells, algae, *Legionella* spp., *K. edwardsii* (K10896), *E. coli* (JM101). (19A) positive control of clone pB2.3. (20A) negative control

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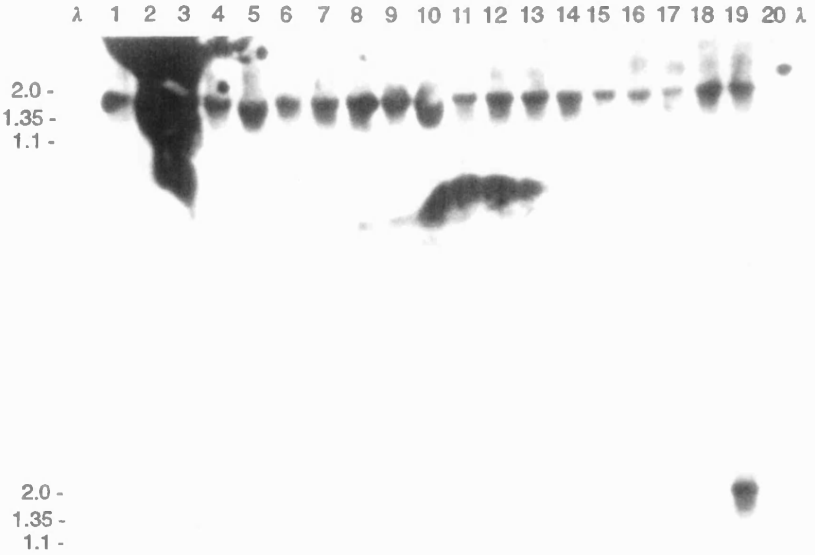

Figure 42 Hybridisation with probe pB2.3 to *N. fowleri* PCR reactions detected on agarose gel electrophoresis in Figure 41

In PCR titration studies with primers p3f and p3r using purified *N. fowleri* (MCM) DNA, an amplification product relating to an initial DNA concentration of 1 pg was detected on agarose gel electrophoresis (Figure 43). Alkaline blotting of the gel and hybridisation of the membrane with probe pB2.3 detected down to 100 fg of DNA (Figure 44).

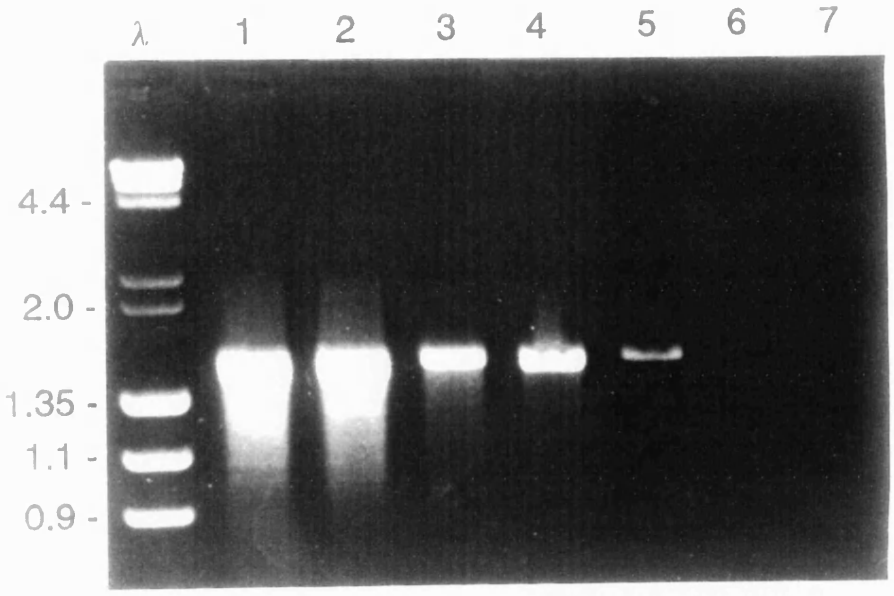

Figure 43 Agarose gel showing PCR sensitivity in detecting *N. fowleri* (MCM) DNA

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest; (1) 10 ng of starting template DNA; (2) 1 ng; (3) 100 pg; (4) 10 pg; (5) 1 pg (6) to 100 fg; (7) negative control

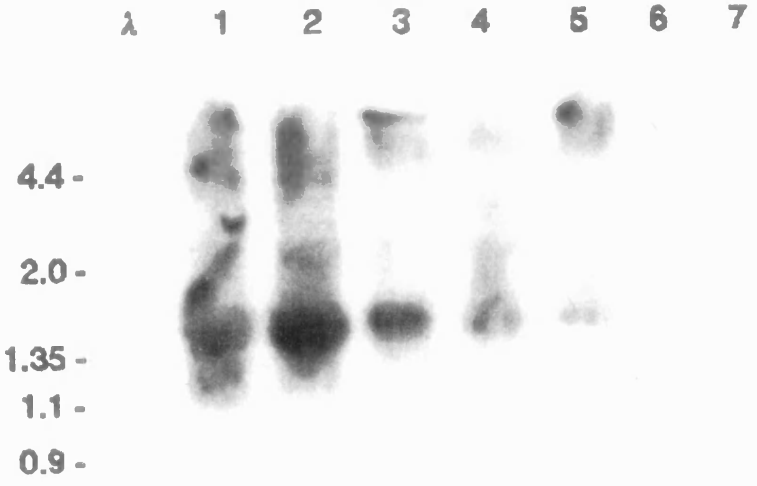

Figure 44 PCR sensitivity in detecting *N. fowleri* (MCM) DNA in conjunction with pB2.3 probe hybridisation

The results of restriction endonuclease digestion of the 1.5 kbp PCR product from all *N. fowleri* strains with Alu I or Hae III is shown in Figure 45 and Figure 46, respectively. Two Alu 1 internal restriction sites were present producing fragments of 840, 480 and 170 bp. With Hae III, one restriction site was present giving two fragments of 1330 and 230 bp.

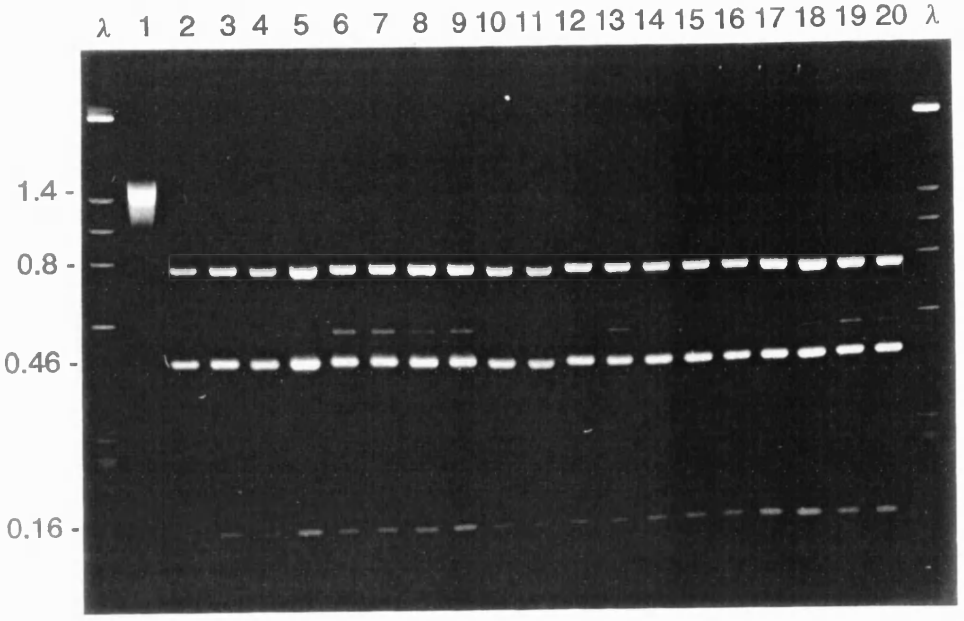

Figure 45 Alu I restriction endonuclease digestion of the *N. fowleri* pB2.3 1.5 kbp PCR product

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(λ) lambda-Hind III/ΦX-174 RF-Hae III digest; (1) undigested PCR product; (2) 2-20 digested PCR product

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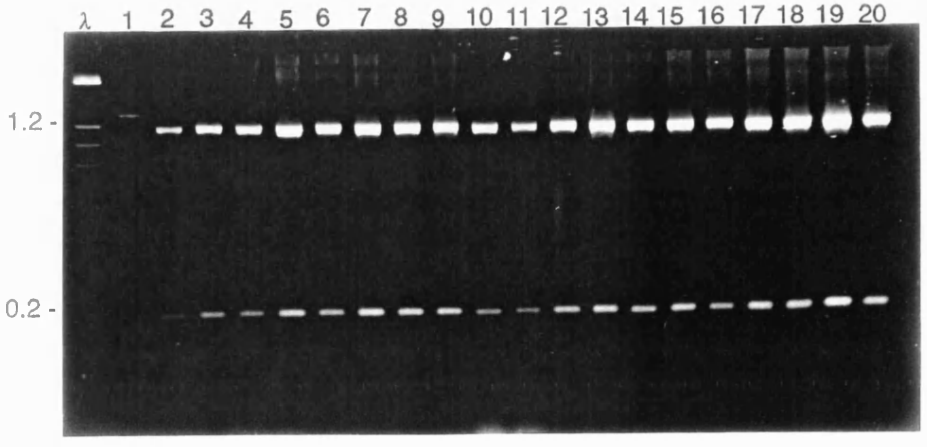

Figure 46 Hae III restriction endonuclease digestion of the *N. fowleri* pB2.3 1.5 kbp PCR product

No amplification occurred with the primer sets designed to amplify the *N. fowleri* DNA clone pB2.2.4 (p4f-gAgCTCggTACCCgATCTCT and p4r-ACCgTTTACACTTggggTggA). Altering the primer annealing temperature to 40°C, 45°C, 50°C, 55°C, 58°C or 60°C and use of different preparations of *N. fowleri* DNA still did not result in PCR amplification. The synthesis of fresh oligonucleotides (Appligene, Durham, England) in which a different forward primer was used ($p4f_2$-CCCgATCTCTAgAgTCgCAATgg) also failed to result in PCR amplification. However, both old and new sets of primers did result in DNA amplification of expected size (~700 bp) when the pB2.2.4 plasmid clone was used as the template.

5.4.3. Discussion

A PCR for the detection of *N. fowleri* was developed with primers derived from partial sequence data of a pUC 18 plasmid clone pB2.3 of chromosomal DNA from the organism. After extensive screening against a wide range of organism DNA, only PCR amplification of *N. fowleri* DNA occurred giving rise to a 1.5 kbp product. Under the conditions described, the PCR could detect as little as 1 pg of purified DNA on agarose gel electrophoresis or 100 fg in conjunction with probe hybridisation. This is equivalent to approximately 6 and 1 trophozoite respectively (Clark, 1990). In organism sensitivity studies, as few as 10 *N. fowleri* trophozoites or cysts resulted in a visible amplification product following 35 PCR cycles. If this was extended to 45 cycles then a detection sensitivity of 1 organism was possible.

The failure of primers constructed to the clone pB2.2.4 to amplify native *N. fowleri* DNA is unclear. This clone has been used successfully as a DNA probe to detect *N. fowleri (4.3. Part I: the development of N. fowleri specific DNA probes*) and successful amplification did occur when the plasmid clone was used as template in the PCR. However, the PCR can be a capricious technique. Usually, problems arise from nonspecific or weak amplification and altering the Taq polymerase buffer (Krawetz *et al*, 1989) or the inclusion of formamide in the reaction (Sarkar *et al*, 1990) can remedy this effect. Reasons for the absence of amplification are less easily addressed. Obviously, the primers must be correct to anneal to the template and the right temperature chosen for this to occur. Here, the primers for pB2.2.4 did amplify the plasmid clone but would not do so with purified genomic *N. fowleri* DNA and are therefore considered appropriate. Nor did the use of different annealing temperatures ranging from 40°C to 60°C result in PCR amplification. Often moving the primers a few base pairs in either direction can solve the problem (Saiki, 1989). However, altering the forward primer in this case had no effect but perhaps the position of reverse primer should also have been moved. Template DNA which is GC rich can also prove difficult to amplify because of difficulties in denaturing fully the DNA. In one study, PCR amplification of such a template was only possible when denaturation was achieved with NaOH (Agarwal & Perl, 1993). Although the limited sequence information available for clone pB2.2.4 does not indicate that it is particularly GC rich, it may be that such regions occur and inefficient denaturation prior to the PCR result in the failed DNA amplification.

5.5. PART III: DEVELOPMENT OF A RAPID DNA EXTRACTION METHOD FOR THE PCR DETECTION OF *N. FOWLERI*

5.5.1. Introduction

Having optimised the PCR for the detection of *N. fowleri* DNA using primer sets to pB2.3, the objective was to proceed to the development of the technique for the identification of the organism from the environment. To this end it was necessary to devise a method for the rapid extraction of DNA from trophozoite or cysts.

Conventional methods for the isolation of DNA from microorganisms involving phenol and chloroform extraction steps are to be avoided as they can generate aerosols. This, together with the several transfer stages involved, can increase the chances of cross contamination in the PCR (Clewley, 1993). In addition, residual phenol in the nucleic acid preparations can degrade the Taq polymerase.

Several methods have been described for the rapid extraction of microorganism DNA for PCR. Simply resuspending the cells in dH_2O and boiling (with or without freeze-thawing cycles) has been found suitable for the bacteria *Legionella* spp. and *Haemophilus influenzae* (Stanbach *et al*, 1989; Bej *et al*, 1991a & 1991b; Jordens, 1993). Resuspending cells in 0.1% diethylpyrocarbonate (DEPC) and heating at 90°C for 5 minutes has been used to prepare DNA from the protozoan parasite *Giardia duodenalis* (synonyms *G. intestinalis*, *G. lamblia*: Mahbubani *et al*, 1991 & 1992). Extraction with 25% Chelex resin has been proposed as a method for the preparation of tissue samples otherwise inhibitory to the PCR (Welsh *et al*, 1991b). Incubation of cells in a Taq polymerase buffer containing proteinase K, followed by heat inactivation of the enzyme, has been used for the isolation of DNA from white blood cells (Higuchi, 1989). In this part of the study, methods for the simple and rapid extraction of *N. fowleri* DNA from trophozoites and cysts for use in the PCR were compared.

5.5.2. Materials and Methods

5.5.2.1. Development of PCR application. *N. fowleri* and *N. lovaniensis* trophozoites were grown on NNA-*K. edwardsii* plates at 37°C. Cysts were produced by prolonged incubation of the cultures.

Several methods were investigated for the rapid extraction of *N. fowleri* DNA from trophozoites and cysts taken directly from NNA-*K. edwardsii*. This involved sweeping a 1 cm^2 area of dense amoeba growth with a disposable bacteriological loop, which was found to recover approximately 1-2 x 10^3 trophozoites, and resuspending the cells in:

i. 60 $\mu\ell$ of dH_2O, placing in a boiling water bath for 10 minutes and chilling on ice.

ii. 60 $\mu\ell$ of 0.1% or 0.2% DEPC, placing in a boiling water bath for 10 minutes and chilling on ice.

iii. 70 $\mu\ell$ of PCR lysis solution (10 $\mu\ell$ of X10 Taq buffer; 1 $\mu\ell$ of 10 mg/ml proteinase K; and dH_2O to 70 $\mu\ell$), overlaying with 50 $\mu\ell$ of sterile mineral oil and incubating at 60°C for 1 hour. The tubes were then placed in a boiling water bath for 10 minutes and chilled on ice (**Appendix 3.25.1**).

Ten $\mu\ell$ of X10 Taq buffer was then added (omitted for method iii), 0.2 mM of each dNTP, 1 μM of each primer and 2.5 U of Taq polymerase to give a final volume of 100 $\mu\ell$. PCR was performed as described in **Part II** except the first cycle of 95°C for 5 minutes was omitted.

5.5.2.2. Sensitivity of the PCR. Serial dilution of *N. fowleri* (MCM) trophozoites or cysts were made so that 10^3-10 organisms were present in 20 $\mu\ell$ of dH_2O. Using microcapillary tube manipulation, 1 trophozoite or cyst was also added to 20 $\mu\ell$ of dH_2O. Following the addition to each tube of 10 $\mu\ell$ of X10 Taq buffer; 1 $\mu\ell$ of 10 mg/ml proteinase K; and dH_2O to 70 $\mu\ell$ the samples were incubated, boiled and processed for PCR amplification as described above in method iii (**Appendix 3.25.2**).

5.5.3. Results

Extraction of DNA from *N. fowleri* by boiling in dH_2O or 0.1-0.2% DEPC did not result in PCR amplification. When purified *N. fowleri* DNA was used in place of trophozoites or cysts, amplification did occur after boiling in dH_2O but still not with the DEPC method. Only DNA extracted by incubating the cells in Taq polymerase buffer containing proteinase K resulted in PCR amplification. However, occasionally amplification failed to occur with this method. This was found to be due to the carry-over of agar from the NNA-*K edwardsii* medium when the trophozoites or cysts were removed with the plastic

inoculating loop. Presumably, the agar is inhibitory to the Taq polymerase. When care was taken to avoid recovering agar when sampling the amoebae, successful PCR amplification invariably resulted.

In the sensitivity studies, down to 10 *N. fowleri* trophozoites or cysts resulted in a visible amplification product following 35 PCR cycles. If this was extended to 45 cycles then a detection sensitivity of 1 organism was possible (results not shown).

5.5.4. Discussion

In the development of the rapid PCR assay for *N. fowleri*, boiling alone was not suitable for the preparation of DNA from trophozoites or cysts. This is likely to be due to the failure to inactivate *N. fowleri* intracellular nucleases that then destroy the DNA. Similarly, boiling in the presence of 0.1-0.2%DEPC was also ineffective. DEPC is a potent nuclease inhibitor and decomposes to ethanol and carbon dioxide on heating. As trace amounts of ethanol does not inhibit Taq polymerase (Gelfand, 1989), this suggests that the DEPC was not fully decomposed by boiling and hence inactivated the Taq polymerase.

Only digestion with proteinase K in Taq polymerase buffer at 60°C for 1 hour, followed by boiling for 10 minutes to inactivate the enzyme, resulted in the isolation of *N. fowleri* DNA suitable for PCR. The Taq polymerase buffer used in the lysis step contains Triton X-100, a nonionic detergent, and may also enhance the release of DNA from the *N. fowleri* cells. Although primarily designed for the extraction of *N. fowleri* DNA from amoebae grown on NNA-*E. coli* or NNA-*K. edwardsii*, the technique was also successfully applied to cells suspended in dH_2O. In this instance, 10 trophozoites or cysts could be detected after 35 amplification cycles and 1 organism after 45 cycles.

Although not extensively investigated the simple DNA extraction method for PCR has been used successfully with several strains of *Acanthamoeba* and *Tetrahymena pyriformis* (CCAP 1640/1w) in the amplification of the small subunit rRNA gene using the universal primers described by Embley (1991).

5.6.PART IV: RAPID DETECTION OF *N. FOWLERI* FROM THE ENVIRONMENT USING THE PCR

5.6.1.Introduction

In the next phase of the study, the suitability of the PCR for the identification of *N. fowleri* from the environment was investigated from samples collected from the thermal springs complex at the Roman Baths.

5.6.2.Materials and Methods

5.6.2.1.Sample site. The overflow of the Great Bath at the Main Drain where the water eventually passes into the River Avon at the Roman Baths complex was sampled. This is shown as point F in Figure 35 of *4.4. Part II: The detection of N. fowleri from the environment using DNA probes.*

5.6.2.2.Processing of samples. Silt material was inoculated on to NNA-*K. edwardsii* and incubated at 44°C as described in *4.4 Part II: The detection of N. fowleri from the environment using DNA probes.* For each isolate of *Naegleria* on NNA-*K. edwardsii*, a 1 cm area of dense trophozoite growth was gently scraped with a disposable 1 $\mu\ell$ bacteriological loop with care taken to avoid recovering any agar. The loop was inoculated in 0.5 ml microcentrifuge tubes containing 70 $\mu\ell$ of ice-cold PCR lysis solution (**Appendix 3.25.1** and overlaid with mineral oil. Tubes were then incubated at 60°C for 1 hour, placed in a boiling water bath for 10 minutes and chilled on ice. If the samples were not to be processed immediately for PCR, the tubes were stored frozen at -20°C. Following the addition of 0.2 mM of each dNTP, 1 μM of each primer and 2.5 U of Taq polymerase, thermal cycling was performed as described in section 5.5 of this section. Besides a standard control of a tube lacking DNA, positive controls using known *N. fowleri* and *N. lovaniensis* trophozoites were processed in the same way.

A second scrape was then made of the primary sampling area on the culture plate using a fresh loop and inoculated into a corresponding well of a second microtitre plate containing 100 $\mu\ell$ of NNA-*K. edwardsii*. The plate was then sealed with clear adhesive film and incubated at 37°C for 2-3 days. Sufficient trophozoites remain at the primary sampling site to establish growth in the well of the microtitre plate. Encystment also occurs during incubation and the plates may then be stored at 4°C for at least several weeks with subsequent viable recovery of the strains.

Aliquots of 20 $\mu\ell$ from the PCR samples were analyzed on a 1.2% agarose-TBE gel at 4 v/cm for 2 hours (**Appendix 3.4**). Ethidium bromide at 0.5 μg/ml was included in the agarose gel and electrophoresis buffer. Where a *N. fowleri* PCR product was detected on the gel, the strain was identified in the corresponding well of the replicate microtitre culture plate and subcultured on to fresh

NNA-*K. edwardsii*. The trophozoites were then adapted to growth in #YPNFH (**Appendix 1.3**) and examined by whole-cell DNA restriction endonuclease digestion to confirm their identity as *N. fowleri* (**Appendix 3.2**). Random isolates of *Naegleria* that were negative by PCR were also examined in this way to confirm their identity as *N. lovaniensis*.

The protocol for the identification of *N. fowleri* from environmental samples by the PCR and subsequent verification by detection of whole-cell DNA RFLPs is shown in Figure 47.

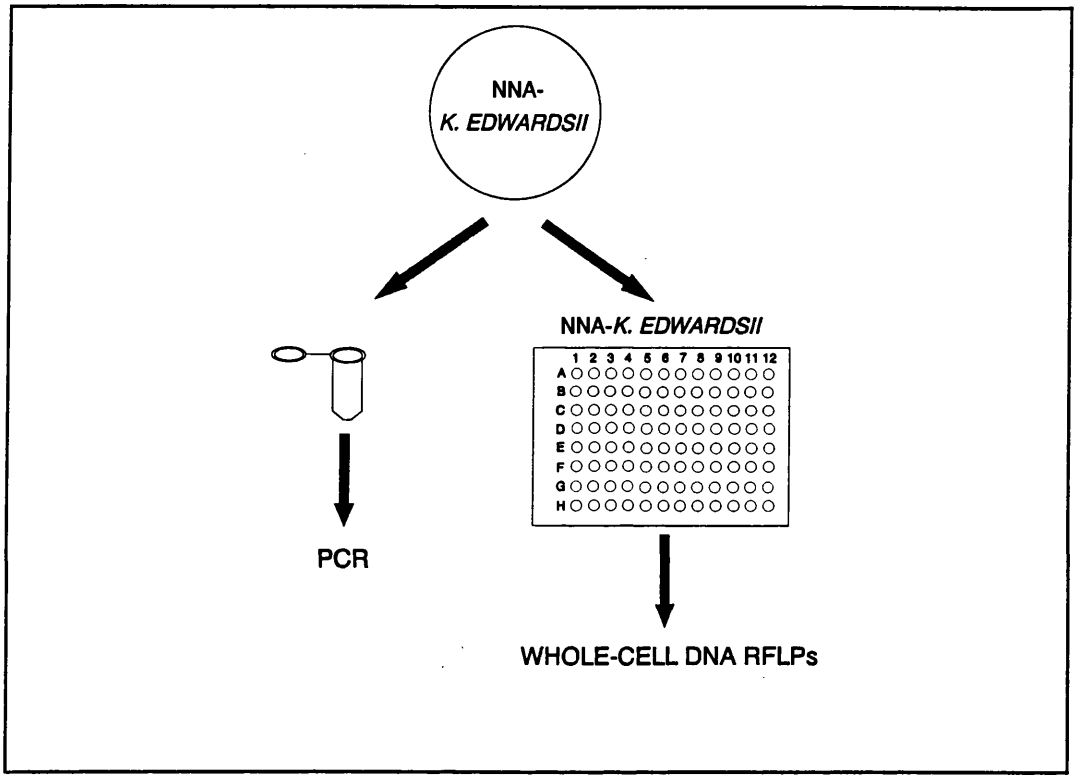

Figure 47 Diagram showing protocol used to identify *N. fowleri* from the environment by PCR

5.6.3. Results

A total of 38 thermophilic *Naegleria* were isolated on NNA-*K. edwardsii* at 44°C incubation. Of these, 4 gave a positive amplification product of expected size by the PCR (Figure 48). Lanes 1 and 2 correspond to the *N. fowleri* and *N. lovaniensis* positive controls, respectively. Analysis of the 4 PCR positive isolates by EcoR I restriction endonuclease digestion of whole-cell DNA gave identical RFLPs typical of *N. fowleri* (Figure 49). Examination of 3 isolates which did not amplify gave EcoR I RFLPs typical of *N. lovaniensis* (Figure 49).

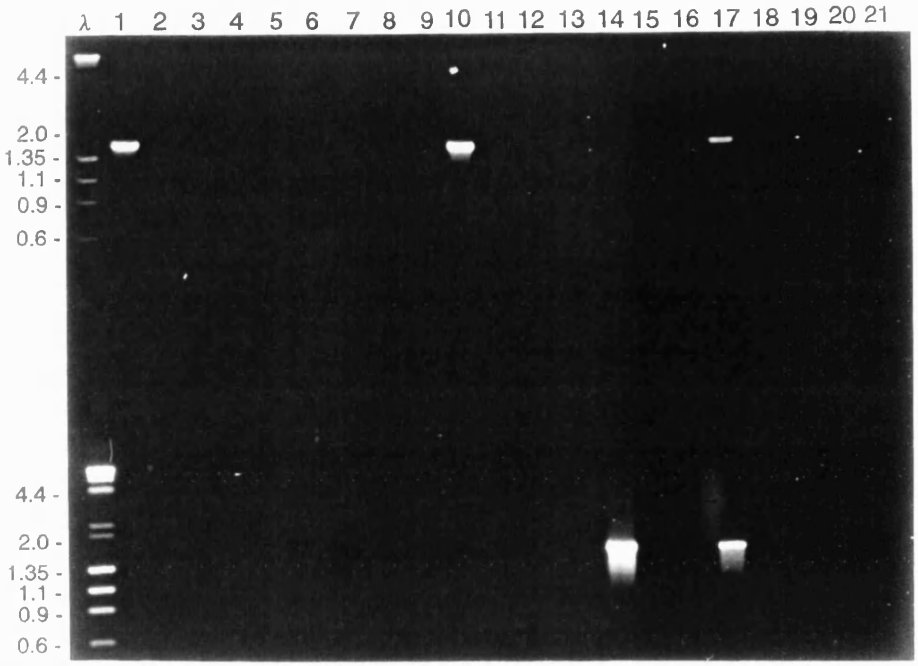

Figure 48 PCR detection of *N. fowleri* from the Roman Baths complex

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest; (1) *N. fowleri* (MCM) positive control; (2) *N. lovaniensis* (C-0490) negative control; (10, 17, 14A, and 17A) Roman Baths isolates of *N. fowleri*

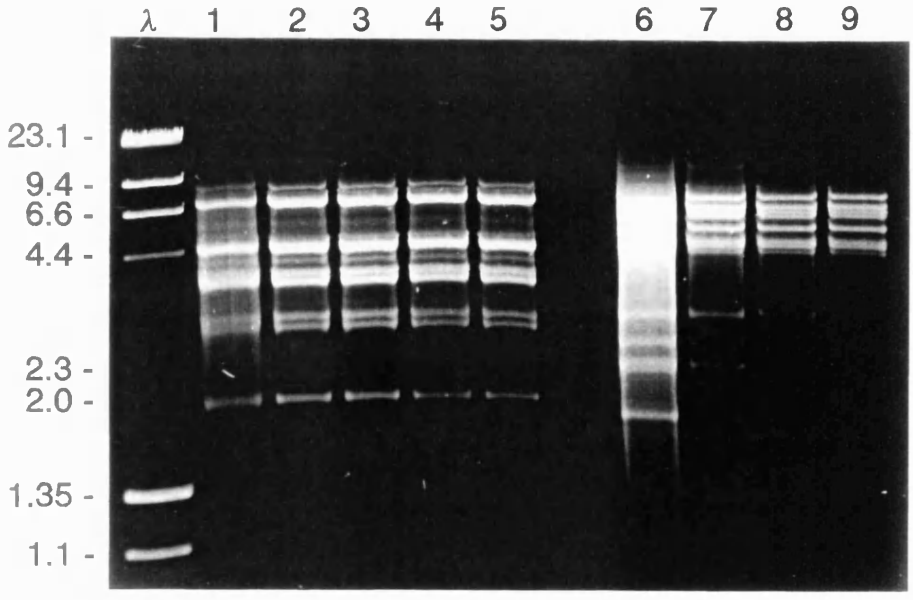

Figure 49 EcoR I whole-cell DNA RFLPs of *N. fowleri* Roman Baths isolates identified by PCR (Figure 47) and also PCR negative *Naegleria* strains

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest; (1) *N. fowleri* (MCM); (2-5) *N. fowleri* detected by PCR in Figure 48; (6) *N. lovaniensis* (C-0490); (7-9) PCR negative isolates

5.6.4. Discussion

In conjunction with the rapid *Naegleria* DNA extraction technique, the PCR was used to identify *N. fowleri* directly following primary isolation on NNA-*K. edwardsii* inoculated with samples taken from the Roman Baths complex. A total of 4 out of 38 isolates were identified as *N. fowleri* by this approach. The identification of the strains was further confirmed by the detection of characteristic EcoR I whole-cell DNA RFLPs (De Jonckheere, 1987c). Following identification of amoebae on the primary NNA-*K. edwardsii* plates, the process of DNA extraction, PCR and gel electrophoresis can be completed in about 6 hours. Numerous samples can be processed simultaneously and, as was noted in the previous section *(4. The development of DNA probes for the identification of Naegleria fowleri)*, the ability to test isolates as soon as they appear on the primary isolation plates may prevent the loss of *N. fowleri* strains through overgrowth of the faster replicating and more predominant *N. lovaniensis* and other thermophilic FLA.

The PCR detection of *N. fowleri* has been previously described. McLaughlin and colleagues (1991) used primers to a repetitive DNA clone from *N. fowleri* to successfully amplify purified nucleic acids and also to detect the organism from crude preparations of infected mouse brains. Low stringency hybridisation at 47°C in the PCR resulted in amplification of DNA in other *Naegleria* spp. besides *N. fowleri*. However, at the higher stringency of 52°C specific amplification of *N. fowleri* DNA was obtained. Sparagano (1993a) also evaluated the PCR amplification of *N. fowleri* DNA using the primers described by McLaughlin and colleagues (1991) but obtained significant cross-reaction with other FLA. When primers derived from the nucleotide sequence data provided by Hu and colleagues (1992) of a potential virulence related gene from *N. fowleri*, specific PCR amplification was found. After 30 and 40 PCR cycles the DNA equivalent of 10 and 1 organism(s) respectively could be detected in conjunction with Southern blotting and hybridisation with an oligonucleotide probe to the amplified region.

The use of the PCR for the rapid identification of *N. fowleri* in artificially seeded water samples has also been evaluated (Sparagano, 1993b). However, the presence of environmental sediment in the water samples was found to be inhibitory to the PCR. Only when amoebae were first isolated by culture on NNA-*E. coli* medium was the PCR able to detect *N. fowleri*. The protocol used required the DNA from the amoebae to be purified by phenol-chloroform extraction and ethanol precipitation which is a lengthy process. In the method developed here, no direct extraction of DNA is required prior to PCR amplification and the fewer manipulation stages involved reduces the chances of cross-contamination that can give rise to false positive reactions (Clewley, 1993).

The PCR detection of *N. fowleri* from the environment is a specific, sensitive and amenable method for the rapid identification of the organism soon after primary isolation. Although environmental samples can contain components inhibitory to Taq polymerase, the direct demonstration of microorganisms in water by the PCR has been reported. Examples include the detection of *L. pneumophila*, coliform bacteria (Mahbubani *et al*, 1990; Bej *et al*, 1991a & 1991b; Jaulhac *et al*, 1993) and *Giardia* cysts (Mahbubani *et*

al, 1992). Rapid methods for the extraction of bacterial DNA from soil samples have also been described (Tsai & Olson, 1991; Jacobsen & Rasmussen, 1992). Evaluation of these methods may, therefore, lend themselves to the direct PCR detection of *N. fowleri* in environmental samples without the need for prior culture isolation.

6. THE ISOLATION OF *NAEGLERIA FOWLERI* FROM ELECTRICITY POWER STATION SITES IN NOTTINGHAM, ENGLAND

6.1. Summary

Naegleria fowleri is found in natural and manmade thermal aquatic sites worldwide. Previous studies from the USA, France and Czechoslovakia have shown that cooling circuits associated with electricity production are suitable environments for the presence of *N. fowleri*. In this study, an environmental survey for the presence of the organism at three coal-fired electricity power stations situated on the River Trent in Nottingham, England was undertaken. *N. fowleri* was isolated from the cooling circuits of two of the three power stations and also the river upstream of the sites. Prior to this study the only isolates of *N. fowleri* in the United Kingdom have come from a fatal case of PAM that occurred in Bath in 1978 and the local hot springs that were the source of the infection. *N. fowleri* may therefore be more widely distributed in this country than has previously been supposed.

6.2. Introduction

Naegleria fowleri causes fatal primary amoebic meningoencephalitis (PAM) in man (Carter, 1970 & 1972). The organism is widely distributed in nature but is more prevalent in thermally enhanced aquatic habitats (John, 1982; De Jonckheere, 1987a). From the natural environment, *N. fowleri* has been isolated from solar heated rivers, lakes, ponds irrigation canals (De Jonckheere *et al*, 1987a; Gupta, 1992; Wellings *et al*, 1977; Lares-Villa *et al*, 1993) and hot springs (Brown *et al*, 1983; Kilvington *et al*, 1991). Manmade environments are also suitable for colonisation by *N. fowleri*. Such sites include chlorinated swimming baths (Kadlec *et al*, 1978; Cursons *et al*, 1979; Gogate & Deodhar, 1985) domestic mains supply water (Anderson & Jamieson, 1972; Esterman *et al*, 1984b), thermal effluents from factories (De Jonckheere *et al*, 1975; Willaert & Stevens, 1976; De Jonckheere & van de Voorde, 1977a; Stevens *et al*, 1977; Cerva *et al*, 1980; Sykora *et al*, 1983), and also the cooling circuits and discharge water of electricity power stations plants (Dive *et al*, 1981; Dive *et al*, 1982; Cerva & Simanoz, 1983; Tyndall *et al*, 1989; Huizinga & McLaughlin, 1990).

The only isolates of *N. fowleri* in the United Kingdom, have come from a fatal case of primary amoebic meningoencephalitis (PAM) that occurred in Bath, England in 1978 and the natural hot springs in the City that were the source of the infection (Cain *et al*, 1980; Kilvington *et al*, 1991). In September 1990, biofilm samples from a water cooling tower were received from Dr John Lee of the Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury. Culture examination for free-living amoebae (FLA) yielded several strains of *N. fowleri*. Subsequent

communication revealed that the samples were from an electricity power station in Nottingham, England. Through the cooperation of the electricity company, an environmental sampling programme was initiated to investigate the distribution of *N. fowleri* in water cooling circuits at three power stations situated on the River Trent in Nottingham. Samples were also taken from the river supplying the power stations to attempt to establish whether this was the source of colonisation of the sites.

6.3. Materials and Methods

6.3.1. Sample sites. Three coal-fired power stations situated on the River Trent at Castle Donington, Ratcliffe and Cottam in Nottingham, England were investigated. The water cooling towers at each site are of similar design, being approximately 350 feet high by 300 feet in diameter at the base. Each cooling tower has a water flow rate of approximately 6.25 million gallons per hour, of which about 30 million gallons per day is taken from the River Trent at each site. The Castle Donington power station is situated furthest upstream on the River Trent, followed by Ratcliffe and then Cottam. The River Soar enters the Trent between the Castle Donington and Ratcliffe power stations,

The Cottam power station was sampled monthly from February 1991 until December 1991 and those at Ratcliffe and Castle Donington from July 1991 until October 1991. Samples of water, mud and silt were taken from entry and exit points to the cooling towers, the ponds within the towers and condenser units. Silt samples were also taken from the banks of the River Trent upstream of the power stations.

6.3.2. Isolation of FLA. Samples were collected into sterile polypropylene containers and dispatched to the Bath Public Health Laboratory for arrival the following day. All samples were processed immediately upon receipt in the laboratory. FLA were isolated by culturing samples on non-nutrient agar plates seeded with a living suspension of *Escherichia coli* (NNA-*E. coli*: **Appendix 1.1**). Water samples were vigorously shaken and 500 ml filtered slowly through a 0.45 μm pore size cellulose acetate membrane over a period of about 5 minutes. Filtration was stopped when 2-3 ml of water remained above the membrane. The water was transferred to a Universal container and the membrane added so that the upper sample surface faced inwards from the walls of the container. After vortexing for 10 seconds, the water was then divided equally over the surface of two NNA-*E. coli* plates and left to stand at room temperature for 4 hours. FLA in the sample attach to the *E. coli* lawn during this time. The excess water was then gently removed with a pipette and the plates placed at 44°C with the lids removed until the surface of the agar was dry. The lids were then replaced and the plates incubated at 44°C in sealed polythene bags for up to 7 days. Plates were examined daily for the presence of discrete plaques in the *E. coli* lawn derived from clonal growths of individual FLA.

This approach permits the quantitative enumeration of FLA as all the organisms in 500 ml of water are concentrated and divided equally over two NNA-*E. coli* plates. Therefore, the number of plaques

obtained from the two plates is equivalent to the number of 44°C tolerant FLA in each 500 ml of water sample.

Mud and silt samples (approximately 1.0 g wet weight) were inoculated directly on to NNA-*E. coli* plates and left to absorb to dryness at room temperature for 1- 2 hours. Plates were sealed and incubated as described above.

6.3.3. Identification of FLA. Plates were examined daily for 7 days for the appearance of discrete growths of amoebal trophozoites. These were then picked with a sterile loop into wells of 96 place flat-bottomed microtitre plates containing 150 $\mu\ell$ of ¼ strength Ringer's solution and incubated at 32°C. The wells were inspected microscopically every hour for up to 4 hours. The genera of FLA were identified from the morphological appearance of the trophozoites and also that of the cyst forms on the culture plates (Page, 1988). *Naegleria* were identified by flagellate formation from the trophozoites under these conditions (Page, 1988).

6.3.4. Identification of thermophilic *Naegleria*. Isolates of FLA producing flagellates were subcultured on to NNA-*E. coli* plates and incubated at 44°C for subsequent identification by cellulose acetate membrane electrophoresis detection of glucose phosphate isomerase as described in *3. Identification of Naegleria species using cellulose acetate membrane electrophoresis of glucose phosphate isomerase.*

6.4. Results

6.4.1. Cottam power station. Regular monthly samples were taken through out the year at the Cottam power station and a total of 183 separate specimens examined. *N. fowleri* was isolated on six occasions in June, July, August, September, October and December. Positive sites were: water entering and leaving the condenser units of the cooling tower; biofilm present on packing material inside the tower; and the cooling tower pond (Table V). *N. fowleri* was also isolated from silt samples of water from the River Trent used to supply the cooling tower ("makeup water intake") in July, August and September (Table V). The results of the survey for the presence of *N. fowleri* and other thermophilic *Naegleria* spp. (*N. lovaniensis* and *N. australiensis*) in river and cooling circuit samples are shown in Figure 50, Figure 51, Figure 52 and Figure 53. The temperature of the sample sites, where recorded, is also shown. Sample points, as defined in Table V, from which *N. fowleri* and other *Naegleria* spp. were isolated during the survey at the Cottam power station are shown in Figure 54.

Of 36 river silt samples, 2 (5.6%) were positive for *N. fowleri* and 4 (11.2%) *Naegleria* spp. This compared to 0 *N. fowleri* and 1 (2.1%) *Naegleria* spp. from 47 river water samples. Using a two-tailed *t*-test analysis and a significance level of 5% ($p<0.05$), the isolation of other *Naegleria* spp. from river silt compared to river water was significantly higher (p 0.0369) but not so for *N. fowleri* ($p>0.05$). For

the cooling circuits, 1 out of 9 (11.2%) silt samples were positive for *N. fowleri* and 3 (33.4%) for *Naegleria* spp. For water samples, 9 out of 88 (10.2%) were positive for *N. fowleri* and 25 (28.4%) for other *Naegleria* spp. No significant difference in the isolation rate between the cooling circuit silt and water samples was found.

The presence of *N. fowleri* in the cooling circuit silt samples compared to the river was not significantly different but was so for other *Naegleria* spp. (p 0.0009). For river and cooling circuit water, the presence of *N. fowleri* and other *Naegleria* spp. in the latter was significantly greater (p 0.0288 and p 0.0013 respectively). Using a one way analysis of variance (ANOVA), no relationship between the temperature of the river or cooling circuit water and the presence of *N. fowleri* or *Naegleria* spp. was found.

With the exception of one condenser unit sample taken in October when 2 organisms /500 ml were detected, the number of *N. fowleri* detected in cooling circuit water samples never exceeded 1 /500 ml. Silt samples from the river and cooling circuit contained only 1 *N. fowleri* /g of wet sample. Biofilm samples taken from packing material inside the cooling tower yielded the largest number of *N. fowleri*. From a single sample set taken in June, six isolates were made from the first layer nearest the base of the tower, 5 from the second and 1 from the third.

N. australiensis and *N. lovaniensis* were the predominant thermophilic *Naegleria* spp. in the river and cooling circuit samples, as summarised in Table VII. Other FLA isolated at 44°C were *Acanthamoeba spp.*, *Hartmannella sp.*, *Willaertia magna* and amoebae that could not be identified by morphological criteria ("FLA").

Table V. *N. fowleri* and *Naegleria* spp. isolated from Cottam power station

					Number of *Naegleria* spp. isolated at 44°C		
Date	Sample site	Sample Nº	Material	Temp (°C)	*N. fowleri*	*N. lovaniensis*	*N. australiensis*
6/91	Unit 1B lower CW* outlet	1	H_2O	27.3	1	0	0
	Unit 3B lower CW outlet	2	H_2O	30.0	1	0	0
	Cooling tower pond	3	H_2O	17.9	1	1	0
	Unit 1B pack	4	Biofilm	-	6	0	2
	Unit 1B pack 2nd layer	5	Biofilm	-	3	0	1
	Unit 1B pack 3rd layer	6	Biofilm	-	1	0	0
7/91	Unit 4B lower CW inlet	7	H_2O	19.4	1	0	0
	Makeup water intake	8	Silt	19.8	1	0	2
8/91	Unit 4B lower CW inlet	9	H_2O	19.0	1	0	2
	Makeup water intake	10	Silt	20.3	1	0	0

Table V. *N. fowleri* and *Naegleria* spp. isolated from Cottam power station

					Number of *Naegleria* spp. isolated at 44°C		
Date	Sample site	Sample Nº	Material	Temp (°C)	*N. fowleri*	*N. lovaniensis*	*N. australiensis*
9/91	Purge water return	11	H_2O	21.0	1	0	9
	Unit 1B lower CW outlet	12	H_2O	30.4	1	0	4
	Makeup water intake	13	Silt	18.9	1	0	0
	Cooling tower pond	14	Silt	20.5	1	0	5
10/91	Unit 2B lower CW inlet	15	H_2O	17.8	2	0	2
	Cooling tower pond	16	Silt	17.8	1	0	6
12/91	Unit 2B lower CW outlet	17	H_2O	23.7	1	0	1

CW* = condenser waterbox

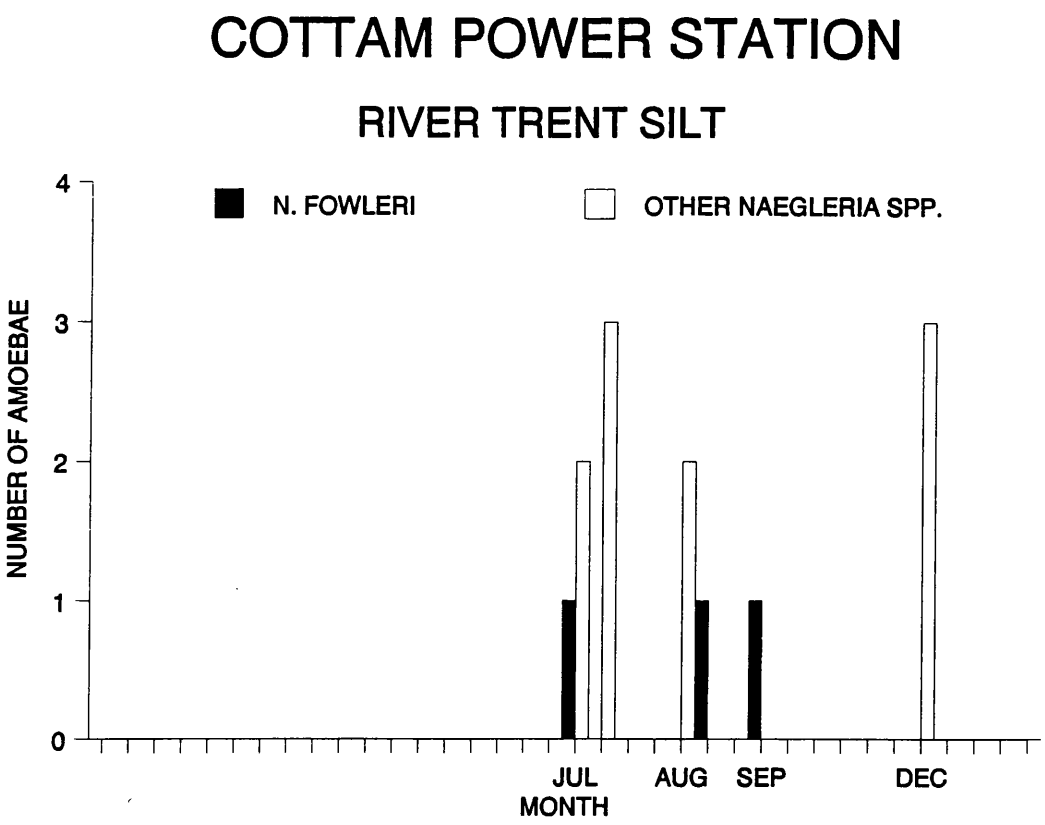

Figure 50 Isolation of *N. fowleri* and other *Naegleria* spp. from river silt at Cottam power station

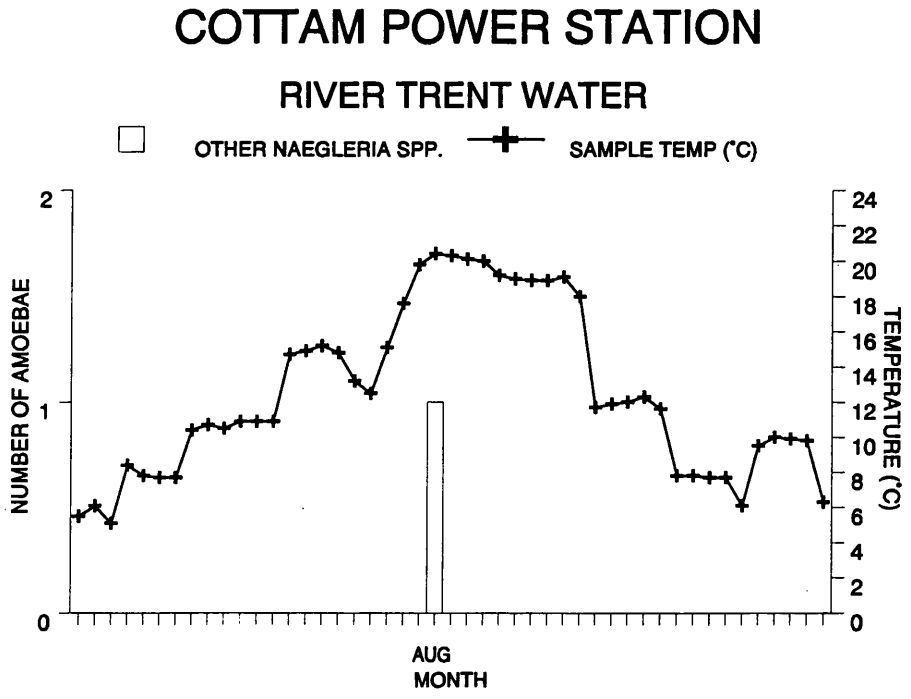

Figure 51 Isolation of *N. fowleri* and other *Naegleria* spp. from river water at Cottam power station

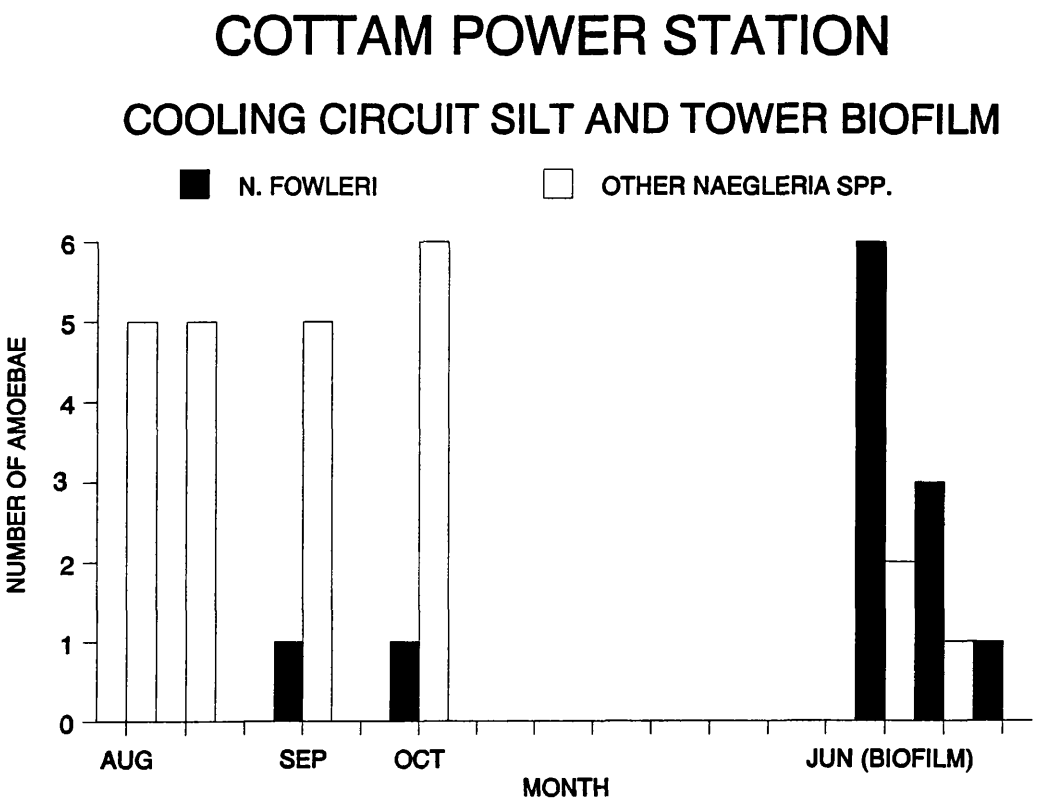

Figure 52 Isolation of *N. fowleri* and other *Naegleria* spp. from cooling circuit silt and biofilm at Cottam power station

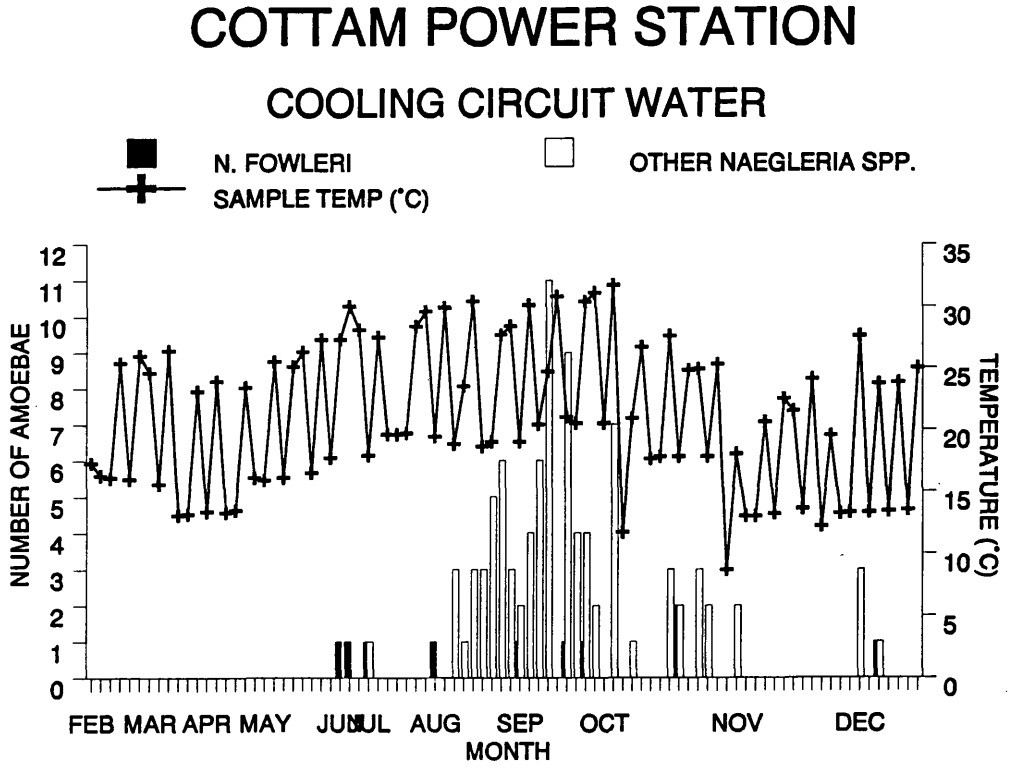

Figure 53 Isolation of *N. fowleri* and other *Naegleria* spp. from cooling circuit water at Cottam power station

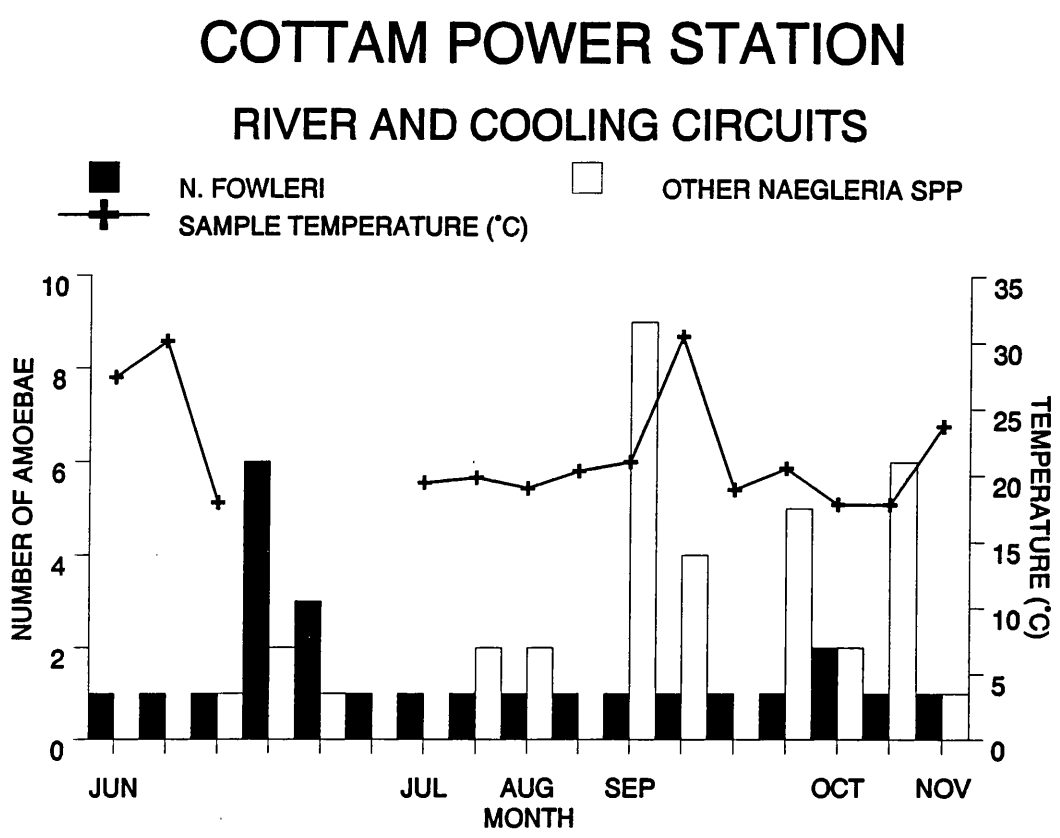

Figure 54 Isolation of *N. fowleri* and other *Naegleria* spp. from river and cooling circuits at Cottam power station

6.4.2. Ratcliffe power station. Four sample sets, comprising 54 separate specimens, were taken from the Ratcliffe power station in July, August, September and October 1991. *N. fowleri* was isolated on three separate occasions in August, September and October. Positive sites were: water entering and leaving the condenser units of the cooling tower; and the cooling tower pond (Table VI). *N. fowleri* was also isolated from silt samples of water from the River Trent used to supply the cooling tower ("makeup water intake") in August, September and October. In addition, isolates were also made from silt samples taken from the river bank upstream of the power station in August and September. The results for the isolation of *N. fowleri* and other *Naegleria* spp. from the river and cooling circuit silt and water samples are shown in Figure 55, Figure 56, Figure 57 and Figure 58. The sample points at the Ratcliffe power station, as defined in Table VI, from which *N. fowleri* and other *Naegleria* spp. were isolated during the survey are shown in Figure 59.

Of 15 river silt samples examined, 3 (20%) were positive for *N. fowleri* and 9 (60%) for other *Naegleria* spp. This compared with 1 *N. fowleri* (5.3%) and 4 (21%) other *Naegleria* spp. from 19 river water samples. As with the findings from the Cottam site, no statistical difference in the isolation rate between the river silt and water samples for *N. fowleri* or other *Naegleria* spp. was found. For the cooling circuit, 2 out of 5 (40%) silt samples were positive for *N. fowleri* and 5 (100%) for other *Naegleria* spp. For cooling water samples, 5 out of 15 (33.4%) were positive for *N. fowleri* and 10 (66.6%) for other

Naegleria spp. Again, no significant difference in the isolation rate for these organisms between the samples was found. Nor was there any correlation between the water temperature and the presence of *N. fowleri* or other *Naegleria* spp. in the cooling circuit or river. In contrast to the findings from the Cottam site, the isolation of *N. fowleri* or other *Naegleria* spp. from the river and cooling circuit silt samples was not significantly different. For river and cooling circuit water, the presence of *N. fowleri* in the latter was significantly higher (p 0.0345) but not so for other *Naegleria* spp.

The number of *N. fowleri* in water samples were 1 /500 ml in August and October, and 2-3 /500 ml in September. Silt samples from the cooling tower pond, and the River Trent yielded slightly higher numbers of *N. fowleri* but never exceeded 3 organisms /g of wet sample.

N. australiensis and *N. lovaniensis* were the predominant thermophilic *Naegleria* spp. in the silt and water samples as summarised in Table VII. Other FLA isolated at 44°C were *Acanthamoeba spp.*, *Hartmannella sp.*, *W. magna* and "FLA".

Table VI. *N. fowleri* and *Naegleria* isolated from Ratcliffe power station

					Number of *Naegleria* spp. isolated at 44°C		
Date	Sample site	Sample №	Material	Temp (°C)	*N. fowleri*	*N. lovaniensis*	*N. australiensis*
8/91	Cooling water from towers	1	H_2O	20.5	1	1	1
	Unit 1A CW* inlet	2	H_2O	20.5	1	0	6
	Unit 1 aux return box	3	H_2O	24.9	1	0	6
	Makeup water intake	4	Silt	21.9	1	0	0
	River Trent gravel bed	5	Silt	21.9	1	0	1
9/91	Cooling water from towers	6	H_2O	16.9	2	0	5
	Purge return to river	7	H_2O	17.3	3	0	1
	River Trent gravel bed	8	Silt	19.2	1	0	1
	Cooling tower pond	9	Silt	19.2	3	0	3
10/91	Unit 1A CW outlet	10	H_2O	27.5	1	0	1
	Makeup water intake	11	Silt	14.0	1	1	1
	Cooling tower pond	12	Silt	16.2	2	0	2

CW* = condenser waterbox

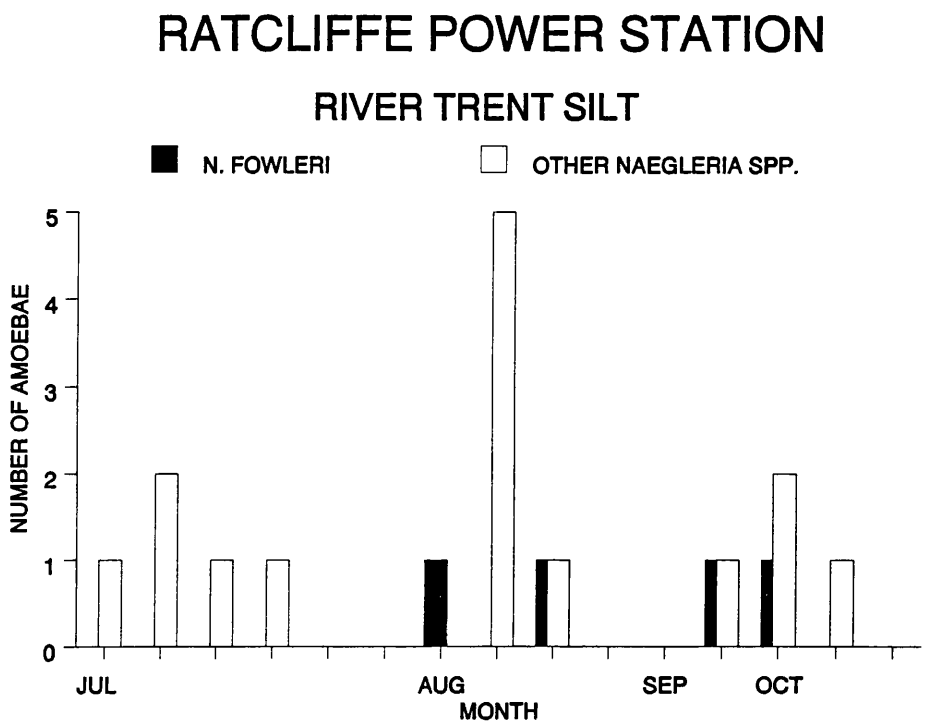

Figure 55 Isolation of *N. fowleri* and other *Naegleria* spp. from river silt at the Ratcliffe power station

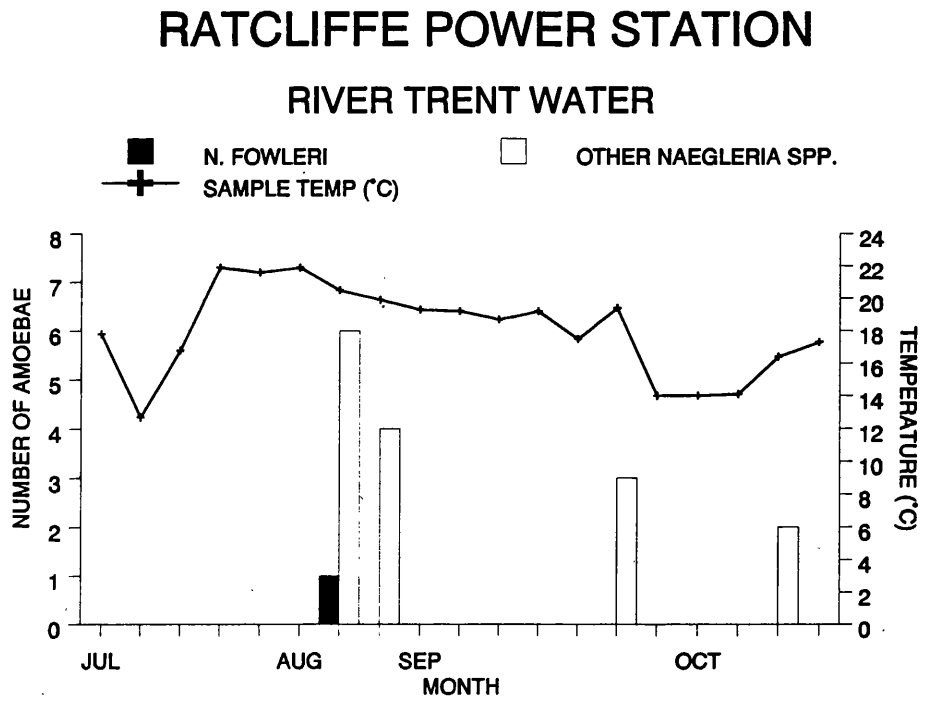

Figure 56 Isolation of *N. fowleri* and other *Naegleria* spp. from river water at Ratcliffe power station

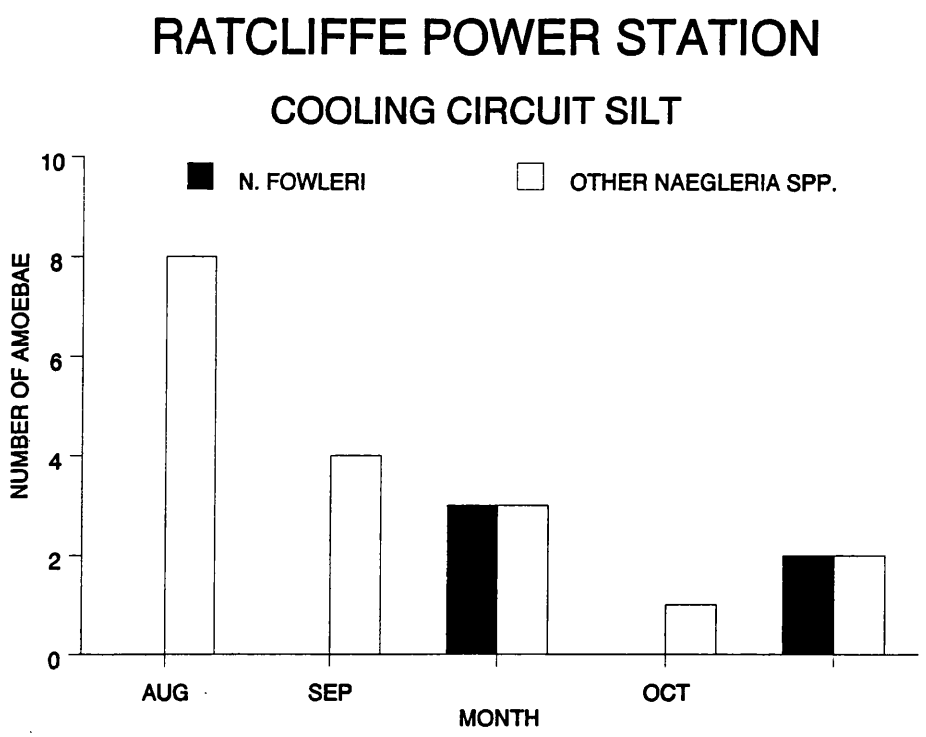

Figure 57 Isolation of *N. fowleri* and other *Naegleria* spp. from cooling circuit silt at the Ratcliffe station

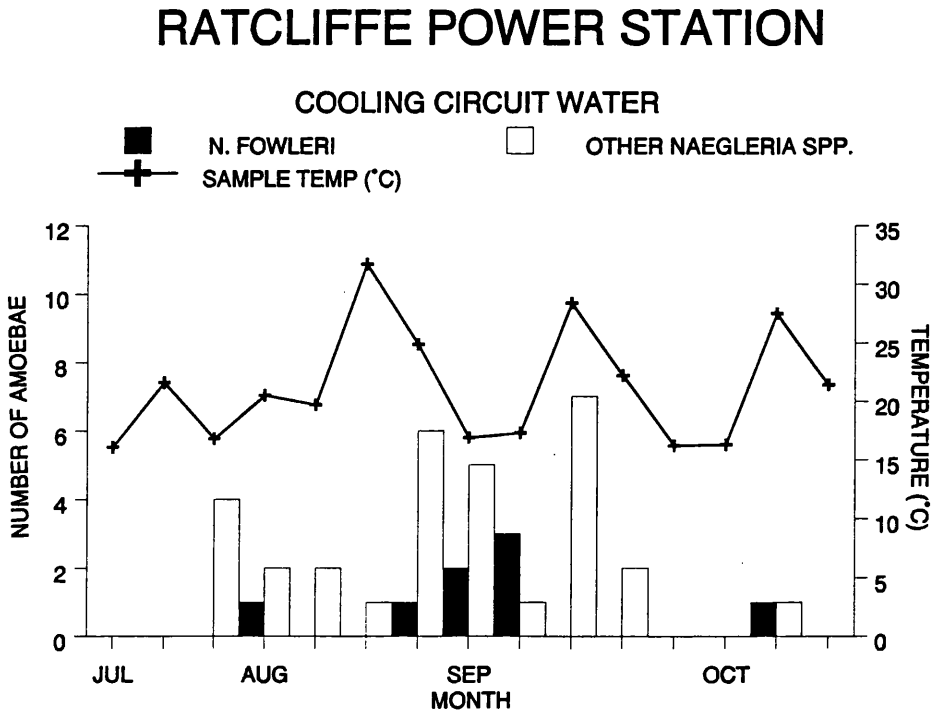

Figure 58 Isolation of *N. fowleri* and other *Naegleria* spp. from cooling circuit water at Ratcliffe power station

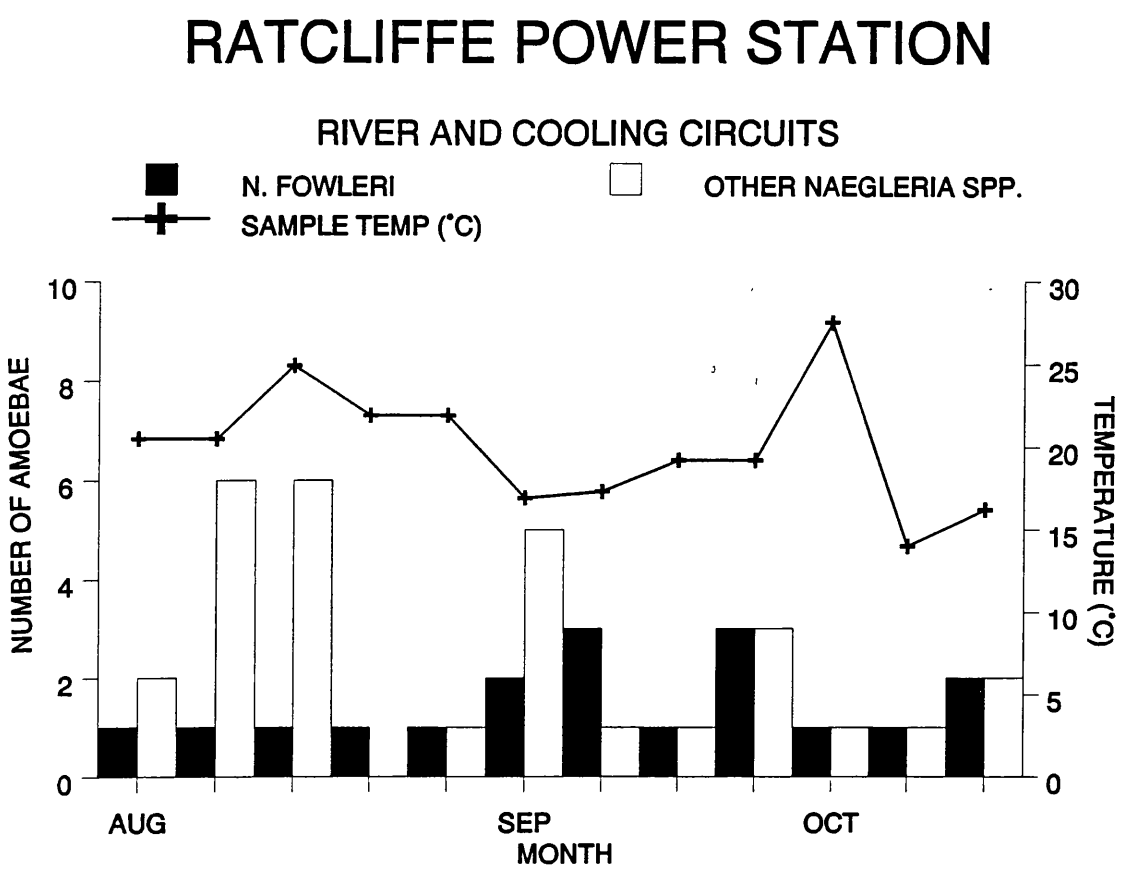

Figure 59 Isolation of *N. fowleri* and other *Naegleria* spp. from river and cooling circuits at the Ratcliffe power station

6.4.3. Castle Donington power station. Four sample sets, comprising 59 separate specimens, were taken from the Castle Donington power station in July, August, September, and October 1991. *N. fowleri* was not isolated from any samples at this site and the only thermophilic *Naegleria* spp. detected was *N. australiensis* (Table VII). The results for the isolation of *Naegleria* spp. from the river and cooling circuit samples are shown in Figure 60, Figure 61 and Figure 62.

No *Naegleria* spp. were isolated from 8 cooling circuit water samples taken in July to October (results not shown). The water temperature during this period ranged from 25.4°C in July to 14.9°C in October. Out of 24 river samples, 3 silt and 3 water specimens (12.5%) were positive for *N. australiensis* only. For the cooling circuits, 2 out of 3 silt samples (66.6%) and 0 out of 8 water samples were positive. These findings were not statistically significant. Other FLA isolated at 44°C were *Acanthamoeba spp.*, *Hartmannella sp.* and "FLA". Overall, the numbers of FLA isolated from the samples were less than those found at the Cottam and Ratcliffe power stations.

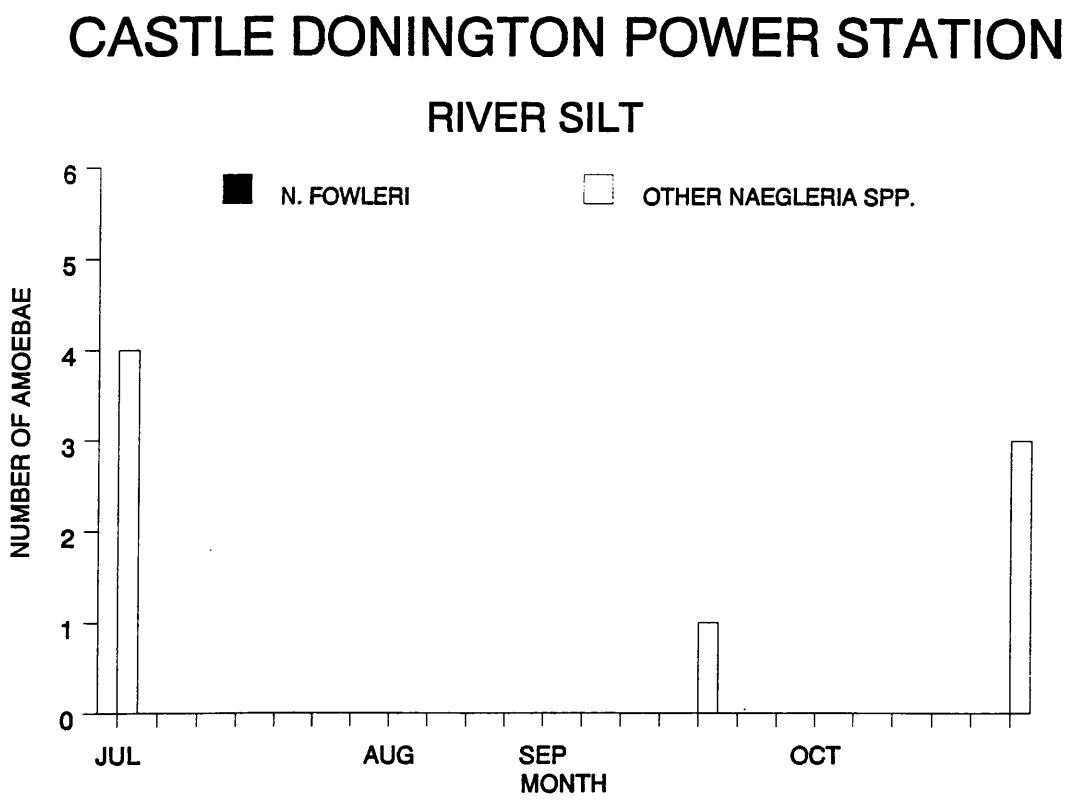

Figure 60 Isolation of *Naegleria* spp. from river silt at Castle Donington power station

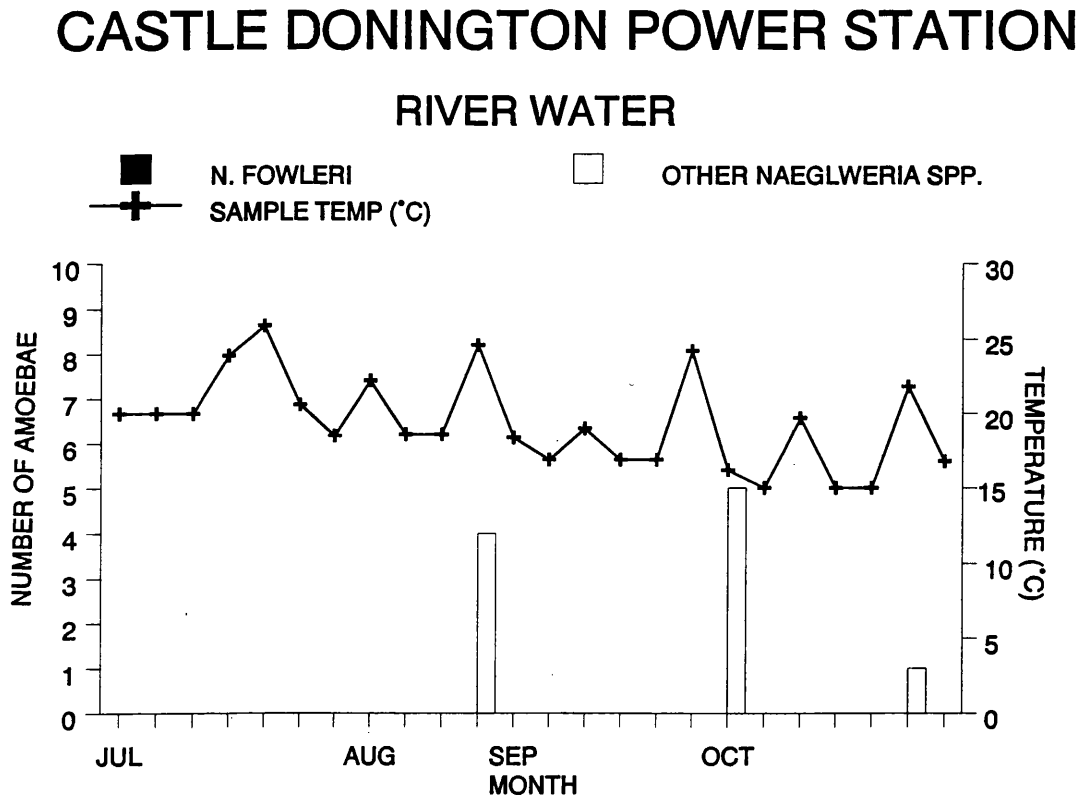

Figure 61 Isolation of *Naegleria* spp. from river water at Castle Donington power station

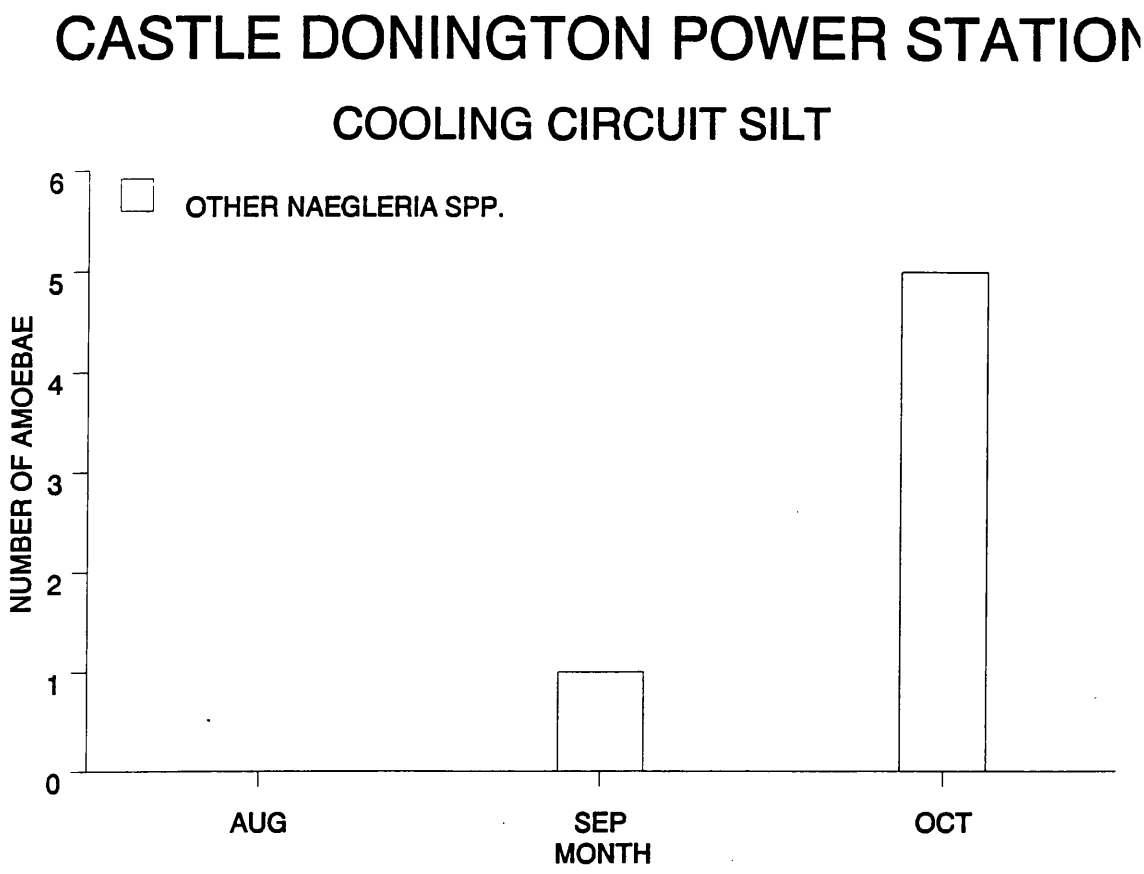

Figure 62 Isolation of *Naegleria* spp. from cooling circuit silt at Castle Donington power station

Table VII. Summary of *N. fowleri* and other thermophilic *Naegleria* made from river and cooling circuit samples

				Isolates made at 44°C			
SAMPLE SITE	SOURCE	MATERIAL	Nº SAMPLES	Nº *N. fowleri*	% *N. fowleri*	Nº *Naegleria* spp	% *Naegleria* spp
Cottam	River	Silt	36	2	5.6	4	11.2
		H_2O	47	0	0	1	2.1
	Cooling circuit	Silt	9	1	11.2	3	33.4
		H_2O	88	9	10.2	25	28.4
		Biofilm	3	3	100	2	66.7
Ratcliffe	River	Silt	15	3	20	9	60
		H_2O	19	1	5.3	4	21
	Cooling circuit	Silt	5	2	40	5	100
		H_2O	15	5	33.4	10	66.6
Castle Donington	River	Silt	24	0	0	3	12.5
		H_2O	24	0	0	3	12.5
	Cooling circuit	Silt	3	0	0	2	66.6
		H_2O	8	0	0	0	0

6.5. Discussion

The findings of this study confirm previous observations from France (Dive *et al*, 1981; Dive *et al*, 1982), Czechoslovakia (Cerva & Simanoz, 1983) and the USA (Tyndall *et al*, 1989; Huizinga & McLaughlin, 1990) on the suitability of cooling circuits associated with electricity production to provide a favourable environment for the presence of *N. fowleri*. Regular sampling at the Cottam power station throughout 1991 suggested a seasonal variation in the presence of *N. fowleri* in the cooling circuits and River Trent as isolates were only made from samples taken in June through to October and once more in December. Although the Ratcliffe power station was sampled only from July to October, *N. fowleri* was detected in samples from the latter three months suggesting a similar pattern of variation may also occur at this site. Water temperatures in the cooling circuits were elevated during the summer and may have resulted in an increased growth of *N. fowleri* and other *Naegleria* spp. This observation is in accord with findings from a similar study in France in which *N. fowleri* was isolated from the cooling pond of an electricity power plant only in the summer months (Dive *et al*, 1981).

Samples from the River Trent taken upstream of the Cottam and Ratcliffe power stations were also positive for *N. fowleri* during the summer months. This may have been due to the release of the organism from the Ratcliffe power station which is located 30 miles upstream of Cottam. However, the origin of the *N. fowleri* isolates above Ratcliffe is unclear as the organism was not detected at the Castle Donington power station located 3 miles further upstream. Although the presence of *N. fowleri* at this site cannot be discounted, it may be that the isolates could have been derived from a separate source of thermal effluent, either between the two power stations or on the River Soar that joins the Trent above Ratcliffe. Whatever the source of these present isolates of *N. fowleri*, the organism must have been present in the river prior to the construction of the power stations in the 1960's and hence colonised the cooling circuits and towers. The growth range of *N. fowleri* has been reported to be $\geq$23 to $\leq$45°C (Griffin, 1972), although the cysts can survive for many months at 4°C (Warhurst *et al*, 1980). As the river water temperature at the Cottam and Ratcliffe sites never rose above 22°C during the survey, the *N. fowleri* isolates are likely to have been derived from the encysted form of the organism. Survival of *N. fowleri* cysts in the river water would also allow the organism to be dispersed over large distances for colonisation of other thermal environments.

Several previous studies have investigated the distribution of *N. fowleri* in thermally altered waters. Stevens *et al*, (1977) found that *N. fowleri* to be absent from seven naturally heated sites with temperatures ranging from 30 to 34°C in the USA. However, the organism was detected from 3 out of 13 sites receiving thermal discharges from electric power plants where the temperatures ranged from 35 to 41°C. In a detailed analysis of a cooling lake associated with a nuclear power plant, Tyndall *et al*, (1989) found the presence of *N. fowleri* to be significantly associated with thermal additions. *N. fowleri* was not, however, detected in river samples taken upstream or downstream of a river supplying the lake. In a similar study from the USA, the presence of *N. fowleri* in a newly created cooling reservoir was also significantly associated with addition of thermal effluent (Huizinga & McLaughlin, 1990).

Prior to this study, the only isolates of *N. fowleri* in the United Kingdom have come from a fatal case of PAM that occurred in Bath in 1978 and the local hot springs that were the source of the infection (Cain *et al*, 1981; Kilvington *et al*, 1991). Regular sampling of this locality over several years resulted in only a few isolates of *N. fowleri* compared with many hundreds of *N. lovaniensis* (Kilvington *et al*, 1991). In contrast, *N. fowleri* was frequently isolated during the summer months from the cooling tower circuits and river at the Cottam and Ratcliffe sites. Griffin (1983) proposed the flagellate-empty hypothesis to account for the sporadic distribution of *N. fowleri* in the environment. The theory suggests that when any human or natural event removes amoebae that usually compete successfully against *N. fowleri*, an advantage is conferred on the species in recolonising the habitat. Therefore, if a thermal site is cleared repeatedly of amoebal and other protozoal competitors, this is advantageous to establishing the presence of *N. fowleri*. Detterline and Wilhelm (1991) found that the presence of *N. fowleri* in thermal aquatic sites in the USA was statistically significant in recently disturbed environments. Accordingly, the large turnover of water and fluctuation in temperature in the cooling circuits of the power station studied here may favour colonisation by *N. fowleri* compared to natural habitats where environmental factors are more constant. At the Castle Donington power station where *N. fowleri* was not isolated, the operation is such that the water cooling towers are rarely required during electricity production. This may provide a more constant environment in which other FLA predominate and so prevent colonisation by *N. fowleri*.

Where quantitative analysis of *N. fowleri* in waters has been undertaken, numbers have ranged from 1-4 per 100 ml from factory discharges in Belgium (De Jonckheere, 1978), to ≥ 1 *N. fowleri* per 10 ml in cooling circuits of a power station in the USA (Tyndall *et al*, 1983). Here, water samples from the cooling circuits at the Cottam and Ratcliffe sites contained only 1-3 *N. fowleri* per 500 ml of water indicating comparatively low levels of colonisation probably because of the lower temperature of the cooling circuits which only on rare occasions reached 30°C. Greatest numbers of *N. fowleri* were recovered from biofilm samples taken from packing material inside the tower with 3-6 isolates per gram of wet material. Pack biofilm samples were only tested on two occasions for *N. fowleri*, once from Ratcliffe power station before this survey and again here from Cottam. All samples were positive for *N. fowleri* and indicates this area to be the site of greatest concentration of the organism in the cooling circuits.

The presence of numerous *N. australiensis* able to grow at 44°C on the NNA-*E. coli* isolation plates was unexpected. *N. australiensis* is reported to not tolerate growth above 42°C (De Jonckheere, 1981) and therefore, the species should have been excluded by the incubation of samples 44°C. Reference strains of the species, including those from the hot springs in Bath, failed to grow in the 44°C incubator used in this study while river and cooling circuit isolates continued to grow when passaged at this temperature. As shown in section *3. Identification of Naegleria species using cellulose acetate membrane electrophoresis of glucose phosphate isomerase,* GPI isoenzyme electrophoresis is a reliable technique for differentiating thermophilic *Naegleria*. The *N. australiensis* strains isolated from the cooling circuits and rivers in this study showed a GPI profile typical of reference strains of the species. Apart from requiring a great deal of additional work in screening the isolates, it was also observed that these *N. australiensis* strains grew

approximately twice as fast as *N. fowleri* on NNA-*E. coli* plates. This may have suppressed the presence of *N. fowleri* during culture isolation and resulted in an underestimation of the frequency and numbers of the species present in the samples. Further examination of these unusual high temperature tolerant strains of *N. australiensis* using additional biochemical and molecular techniques is indicated.

N. fowleri PAM is acquired by intranasal inoculation of contaminated water, usually whilst bathing (John, 1982; Martinez, 1987; Marciano-Cabral, 1988; Warhurst, 1985). Therefore, the risk to workers from the presence of *N. fowleri* in the power station cooling circuits must be considered minimal. *N. fowleri* is sensitive to 1-2 mg/L free-available chlorine and other water treatment disinfectants (De Jonckheere & Van de Voorde, 1976; Kilvington, 1990) and in one study, chlorination to 2 mg/L throughout a cooling circuit reduced the presence of *N. fowleri* 50-100 fold. However, samples taken six months later showed *N. fowleri* numbers comparable to the pretreatment levels (Tyndall *et al*, 1983). Chlorination of the water cooling towers at the power stations investigated here would not be a practical proposition because of the difficulty in achieving amoebacidal concentrations throughout the large systems and subsequent ecological damage resulting from the release of the treated water into the environment. Therefore, the presence of *N. fowleri* in the cooling circuits and towers of the power stations studied here must be accepted as part of the natural ecology of these thermal aquatic sites.

The isolation of *N. fowleri* from the Cottam and Ratcliffe power stations and the River Trent suggests that the organism may be more widely distributed in manmade thermal aquatic sites and possibly ambient waters in the United Kingdom than has previously been supposed. Although the symptoms of PAM may be mistaken for bacterial meningitis (Dos Santos, 1970), the disease is rare. In the United Kingdom only one confirmed and four suspected cases having been reported (Symmers, 1969; Apley *et al*, 1970; Cain *et al*, 1981). PAM cases have been reported from Belgium (Jadin, 1971) and Czechoslovakia (Kadlec, 1987) in persons swimming in canals receiving thermal effluent from factories. Also in Czechoslovakia, 16 cases of PAM were associated with an indoor swimming pool filled with river water which was heated and chlorinated (Cerva & Novak, 1968; Kadlec *et al*, 1978). The possibility of future cases of PAM in the United Kingdom must not be ignored and recreational activity in thermal water discharged from power stations and factories should be prohibited.

7. MOLECULAR DNA TYPING OF *N. FOWLERI* STRAINS FROM THE NOTTINGHAM POWER STATIONS AND OTHER GEOGRAPHIC LOCALITIES

7.1. Summary

Naegleria fowleri from the Nottingham power stations were compared to other strains of the species from various geographic localities worldwide by their restriction fragment length polymorphisms (RFLPs) detected directly on agarose gel electrophoresis or in conjunction with hybridisation using a repetitive element clone of *N. fowleri* chromosomal DNA, pB2.2. Digestion with Bgl II gave agarose gel RFLPs that differentiated the strains into two distinct groups (I and II). Group I comprised strains from the Bath, Belgium, France, Czechoslovakia, Hong Kong and the USA. Group II contained strains from Australia, New Zealand, the Nottingham power stations and also some isolates from a power station in France. However, hybridisation with pB2.2 probe detected RFLPs which subdivided these two groups. Within group I, strains from Bath, and some from the USA formed the subgroup IA, and the remainder subgroup IB. This subgroup also included two strains from Australia and New Zealand. Within group II, the Nottingham and French power station strains formed a subgroup IIA, due to a unique ~2.4 kbp RFLP, separate from the remaining Antipodean strains assigned to subgroup IIB. When the Bgl II and pB2.2 RFLPs were examined together, the same subgroups were maintained except that the two Australian and New Zealand strains of subgroup IB formed a separate subgroup IIC within the Antipodean-power station complex of group II. The detection of agarose gel and DNA probe RFLPs is a powerful approach to the differentiation of *N. fowleri* strains and enabled the identification of a subgroup of the species so far isolated found only in power stations in France and England. As this subgroup was not identified in *N. fowleri* from the natural hot springs in Bath, it seems likely that the two sites have been colonised separately. This further suggests that the organism may be more widely distributed in this country than has previously been supposed.

7.2.Introduction

Members of the genus *Naegleria* contain an extrachromosomal ribosomal DNA (RDNA) plasmid and mitochondrial DNA present in high copy number within the cell (Clark and Cross, 1987 & 1988). Analysis of whole-cell DNA preparations enables the detection of restriction fragment length polymorphisms (RFLPs) directly on agarose gel electrophoresis and represent digestion products from both the rDNA and mitochondrial DNA (*4. The development of DNA Probes for the Identification of Naegleria fowleri*). The detection of such RFLPs has been shown to be a powerful technique for the differentiation of *Naegleria* species (De Jonckheere, 1987c; McLaughlin, *et al*, 1988; Milligan & Band, 1988; Clarke *et al*, 1989) and also for the detection of inter-strain differences within *N. fowleri* that appear to correlate with their geographic origin. Strains from the Antipodes were found to be distinct from those in Europe, whilst

within the USA either profile occurred (De Jonckheere, 1988b; Clark *et al*, 1989). Recently, analysis of *N. fowleri* isolates from an electricity power station in France and the Moselle river associated with the site revealed an Antipodean RFLP type which had not previously been detected in Europe (Pernin & De Jonckheere, 1992). The presence of this type of N. fowleri was considered to be due to recent introduction, probably as a result of human intervention.

At the time of the Nottingham power station surveys, the DNA probes or PCR for the detection of *N. fowleri* had not been developed. Accordingly, the strains were identified using cellulose acetate membrane electrophoresis of glucose phosphate isomerase (GPI). In this part of the study these strains were re-examined for their whole-cell DNA RFLPs, detected directly on agarose gel electrophoresis and also in conjunction with hybridisation using a repetitive element clone of *N. fowleri* chromosomal DNA.

7.3. Materials and Methods

7.3.1. *N. fowleri* strains and DNA isolation. *N. fowleri* strains, identified by GPI isoenzyme profile (see *3. Identification of Naegleria species using cellulose acetate membrane electrophoresis of glucose phosphate isomerase*), were recovered from liquid nitrogen storage and cultured in #SCGYM or #YPNFH (see *2. The Culture and Cryopreservation of Naegleria*). *N. fowleri* strains used in the study and their geographic origins are listed in Table VIII.

N. fowleri whole-cell DNA was isolated as described in section *4. The development of DNA Probes for the Identification of Naegleria fowleri* and detailed in **Appendix 3.2.** Approximately 3 μg of whole-cell DNA from the strains were digested with 10-20 U of the restriction endonucleases Bcl I, Bgl II, BstE II, EcoR I, Hae III, Sal I or Sau3A I (**Appendix 3.3**) using appropriate reaction buffers supplied by the manufacturer (Northumbria Biologicals Ltd, Northumbria, England). Samples were digested for 3 hours at 37°C except for BstE II and Bcl I where incubation was at 60°C and 50°C respectively. Samples were loaded on to horizontal 0.7% agarose gels (20 cm x 20 cm x 0.5 cm) prepared in TBE buffer. DNA standards of λ-Hind III/ΦX-174 RF-Hae III digests (Pharmacia LKB Ltd, Milton Keynes, England) were included as size markers. Gels were stained with 1 μg/ml ethidium bromide in dH_2O for 1 hour, destained in dH_2O for 20 minutes and photographed under shortwave UV transillumination using Polaroid 665 film and a Kodak Wratten #23A orange filter (**Appendix 3.4**).

7.3.2. DNA probe hybridisation. Gels were blotted on to Hybond N nylon membranes by the alkaline transfer method (Reed and Mann, 1985) according to the manufacturer's protocol (Amersham, Buckinghamshire, England) as detailed in **Appendix 3.19.** Membranes were exposed, DNA side down, to UV light from a transilluminator for 5 minutes to fix the DNA. The membranes were hybridised with pUC 18 plasmid subclones of a *N. fowleri* (MCM) DNA genomic DNA library constructed in the λ phage vector EMBL3 as detailed in section *4. The development of DNA Probes for the Identification of Naegleria*

fowleri. The probes were, pB2.2, a 6.1 kbp fragment containing a repeated element, pB2.3, a 1.2 kbp fragment and pB2.2.4 a 0.8 kbp fragment. In addition a 4 kbp Sal I insert from a 20 kbp clone (A2.4) from the λ phage library was also tested.

Plasmid and the λ phage clones were digested with the appropriate restriction endonuclease and separated on 0.7% agarose TAE gels. The DNA fragment was purified by absorption on to silica powder (**Appendix 3.15**) and labelled with 5'-[α-^{32}P] deoxycytidine triphosphate using the oligolabelling (random primer) method (**Appendix 3.11**). One ng of λ phage DNA was included in the labelling reaction to hybridise with λ-Hind III bands of the gel size markers and so indicate their relative positions on the autoradiograph. Prehybridisation and hybridisation of membranes was performed at 65°C in "Westneat buffer" (Westneat *et al*, 1988) as described in **Appendix 3.12**. After washing twice at room temperature for 15 minutes in 2XSSC-0.1% SDS; 15 minutes in 2XSSC-0.1% SDS at 65°C; and 30 minutes in 1XSSC at 65°C the membranes were exposed on to Fuji RX medical X-ray film in autoradiography cassettes (Genetic Research Instrumentation Ltd) with intensifying screens. Typical exposure times were 3-5 days at -70°C. Films were developed in Kodak D19 developer for 5 minutes, rinsed briefly in tap water, fixed in Kodak 'Unifix' for 5 minutes and finally rinsed in tap water for 15 minutes and air-dried.

7.4. Results

Restriction endonuclease digestion of *N. fowleri* whole-cell DNA preparations enabled RFLPs to be detected directly on agarose gel electrophoresis. Hybridisation of membrane transferred DNA from these gels with the *N. fowleri* specific DNA probes pB2.2.4 or pB2.3 detected one or two homologous RFLPs in all strains. Similarly, hybridisation with the 4 kbp Sal I insert (A2.4) from a 20 kbp clone from the *N. fowleri* λ phage library resulted in only a few RFLPs, common to all strains. These findings are not shown as they offered no discrimination between the *N. fowleri* strains. In contrast, the repetitive element clone (pB2.2) detected numerous additional RFLPs enabling the further differentiation of the strains.

For digests with EcoR I, *N. fowleri* from the power stations and river were found to be identical to those from the Antipodes and NF-124 from the USA by the presence of a unique DNA band of ~3 kbp visible directly on the agarose gel. Strains from the Bath hot springs, Belgium and the remainder from the USA had a doublet banding at this region. Strain MSM from New Zealand was also found to have a unique ~1 kbp band absent from all others (Figure 63). The pB2.2 probe RFLPs were identical for all the strains. The results are not shown but were in common to the findings for this enzyme-probe combination used with *N. fowleri* strains studied in section *4. The development of DNA Probes for the Identification of Naegleria fowleri*.

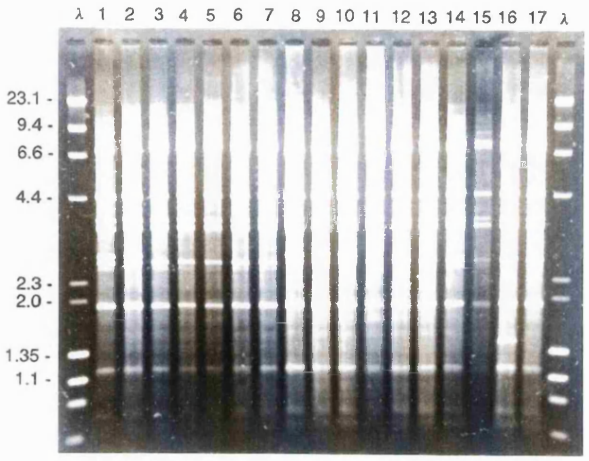

Figure 63 *N. fowleri* EcoR I RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

With Hae III, the power station isolates were also found to resemble those from the Antipodes and NF-124 from the USA by the presence of a ~1.8 kbp band that was absent from the rest (Figure 64). However, the power station strains, NF-124 and Ng 060 from Australia lacked a ~2.5 kbp band present in the other Antipodean isolates. A somewhat poor autoradiograph of the pB2.2 hybridisation reaction was obtained which made interpretation difficult. However, strains from the Antipodes and NF-124 from the USA were found to have a unique band of ~1.5 kbp (Figure 65). All the other strains, including those from the power stations, appeared similar.

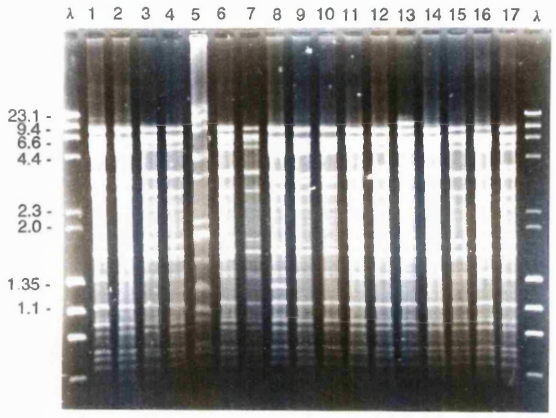

Figure 64 *N. fowleri* Hae III RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

Figure 65 *N. fowleri* Hae III-pB2.2 RFLPs

For BstE II, the power station and Antipodean strains were identical and differed from the others by the presence of a of ~7 kbp band (Figure 66). Here, strain NF-124 from the USA was identical to other isolates from the USA. Hybridisation studies gave similar RFLPs for all the strains except MSM from New Zealand which had a triplet of bands at ~9 kbp where the others had a doublet (Figure 67).

No differences between the strains was found with Bcl I (Figure 68). Again the autoradiograph was of poor quality and although numerous RFLPs were indicated, clear differentiation of the strains was not possible (Figure 69).

Sau3A I digestion resulted in numerous bands which could not be resolved discreetly on the agarose gel (Figure 70). With the probe hybridisation, strains NHI, MSM and 1518/3 showed only degraded DNA and did not allow the detection of RFLPs (Figure 71). However, differences in RFLPs were observed between the other strains. The power station strains were differentiated by the presence of a double band of ~2.3 kbp where a singlet occurred in the rest. The Bath hot springs strains resembled HB-1 and CDC:0487:1 from the USA due to a unique ~3 kbp band. NF-124 from the USA, Ng 060 from Australia and KUL from Belgium were identical. Strains Carter 69 and 1518/4 were characterised by a ~2.8 kbp band.

Conversely, Sal I gave only a few bands, the relative position of which was common to all strains (Figure 72). Probe hybridisation, showed the power station strains to resemble those from Australia by the presence of a unique ~3 kbp band (Figure 73). The other strains, including those from New Zealand, were indistinguishable.

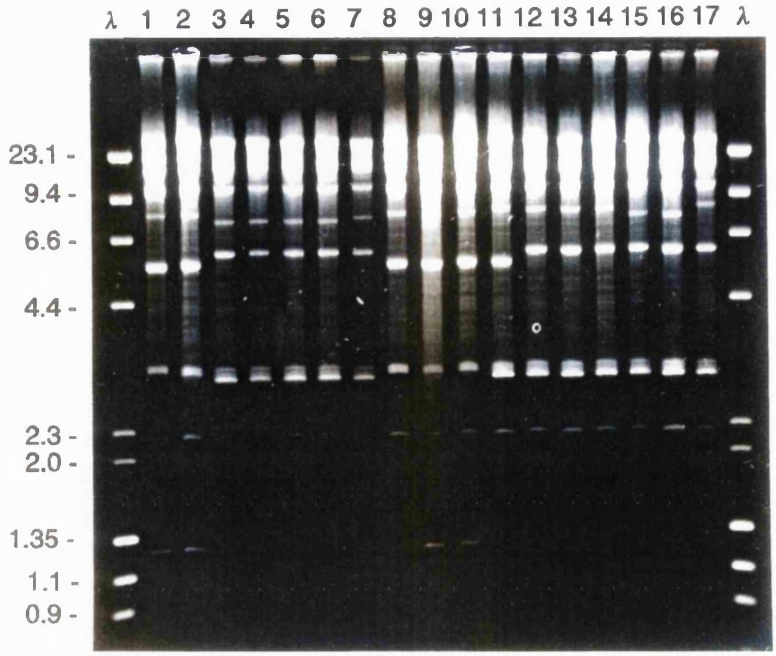

Figure 66 *N. fowleri* BstE II RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

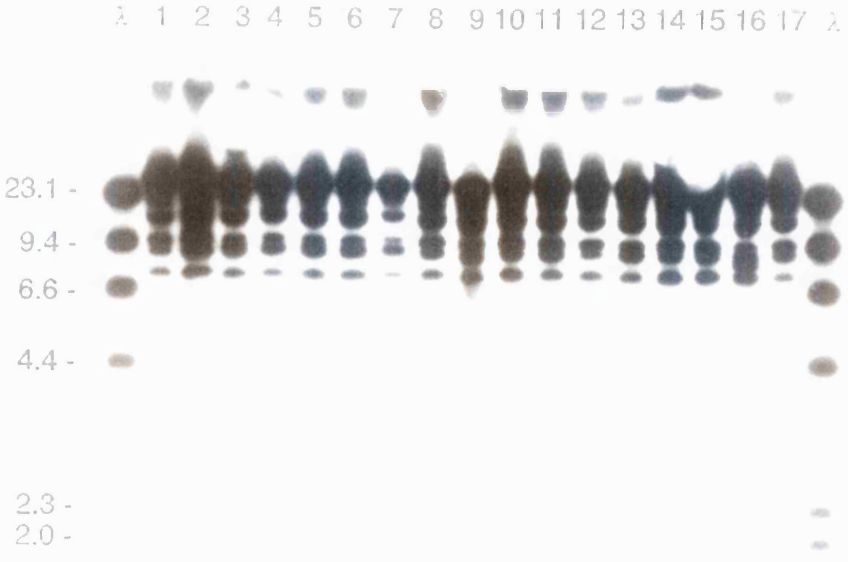

Figure 67 *N. fowleri* BstE II-pB2.2 RFLPs

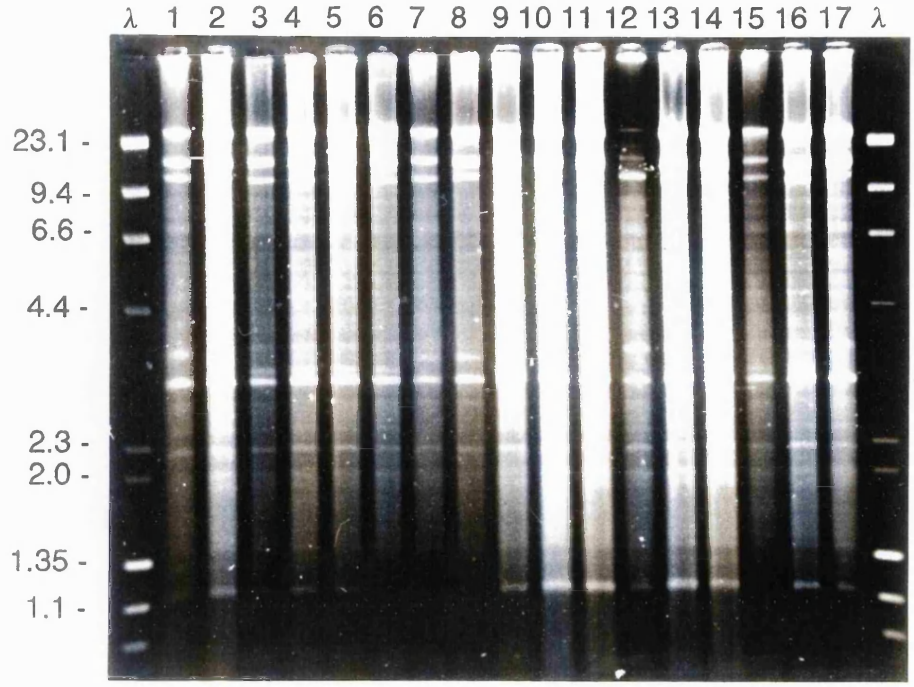

Figure 68 *N. fowleri* Bcl I RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

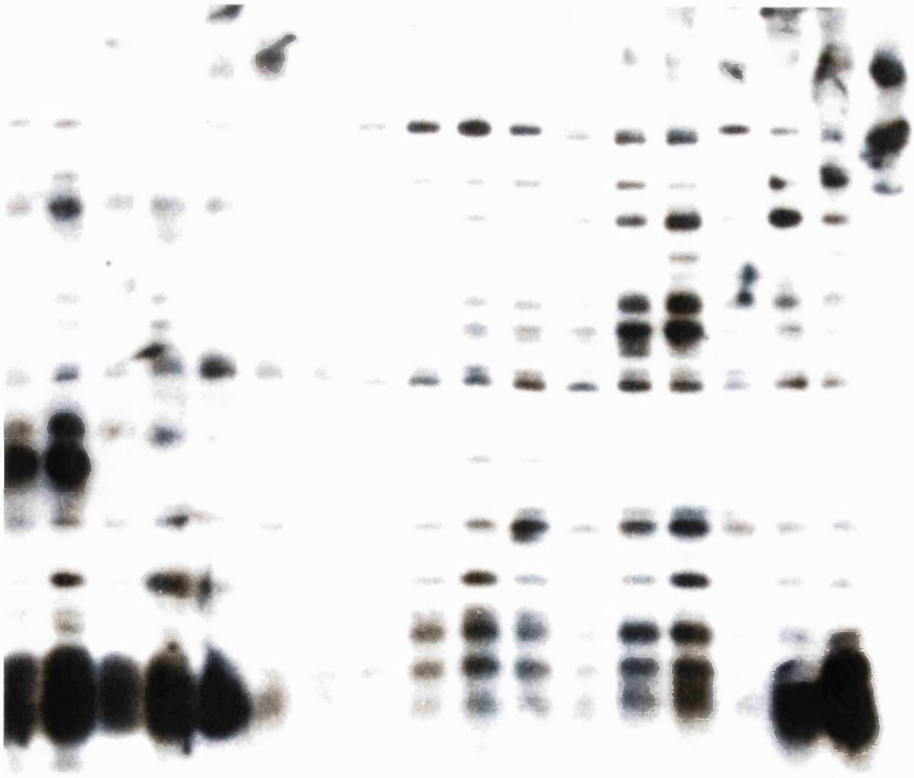

Figure 69 *N. fowleri* Bcl I-pB2.2 RFLPs

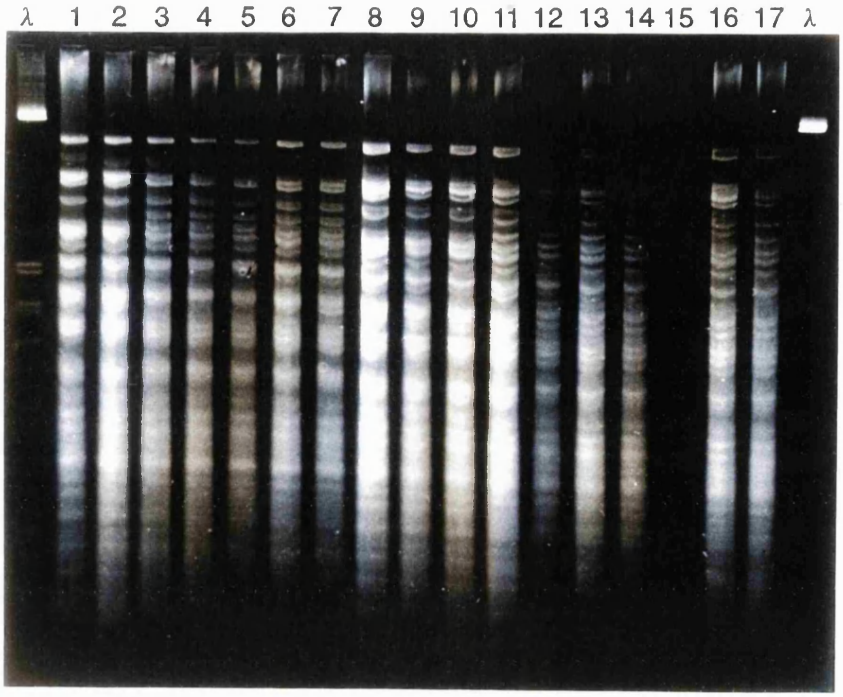

Figure 70 *N. fowleri* Sau3A I RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

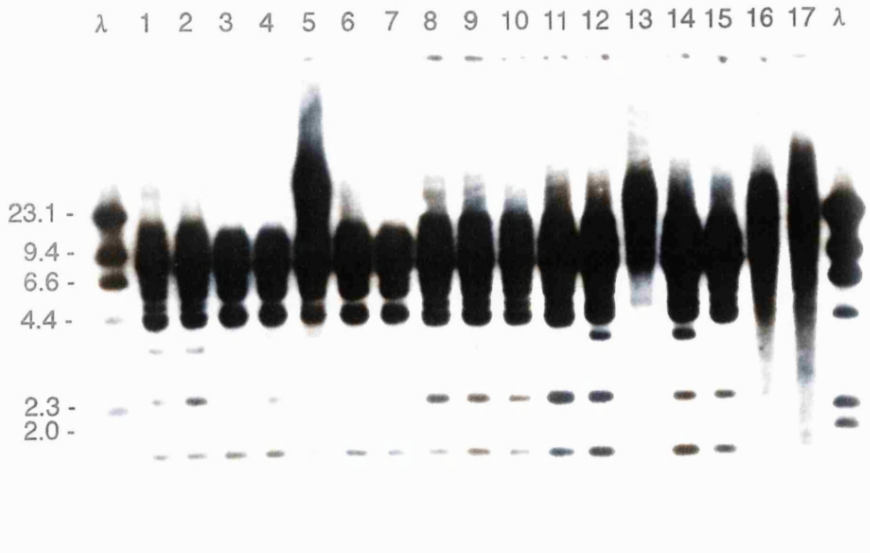

Figure 71 *N. fowleri* Sau3A I-pB2.2 RFLPs

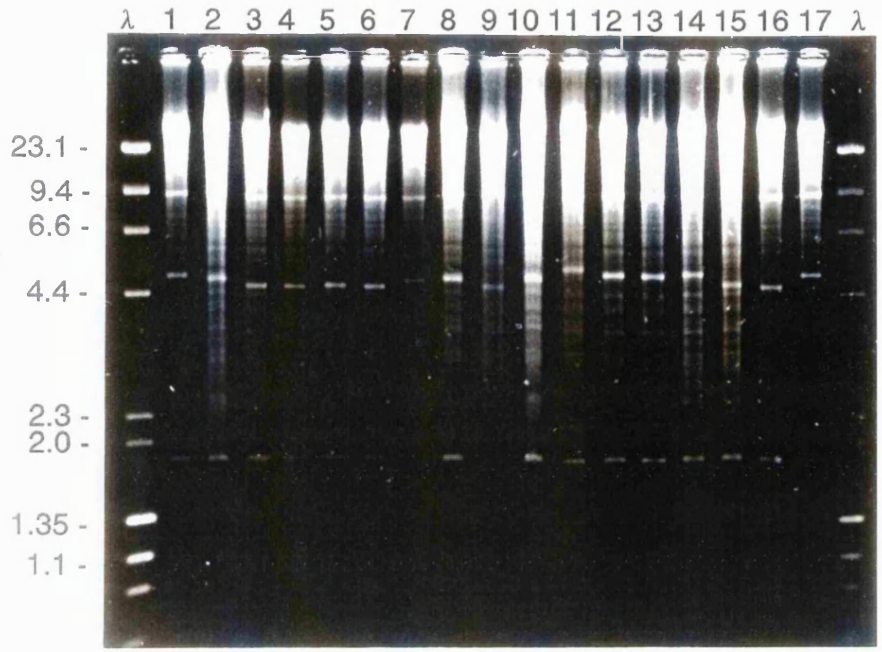

Figure 72 *N. fowleri* Sal I RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

Figure 73 *N. fowleri* Sal I-pB2.2 RFLPs

For Bgl II digestion, the power station strains were identical to those from the Antipodes and were characterised by a unique ~1.9 kbp band (Figure 74). The other strains from the Bath hot springs, Belgium and the USA were identical and showed a characteristic band of ~1 kbp. Hybridisation with the pB2.2 probe identified a ~2.4 kbp RFLP in the power station strains which was not present in any other isolates, including those from the Antipodes (Figure 75). All strains from the Bath hot springs, and CDC:0487:1 and HB-1 from the USA were similar and were characterised by a ~3.5 kbp band. Strain KUL from Belgium and NF-124 from the USA resembled Ng 060 from Australia and MSM from New Zealand by lacking a ~3.8 kbp present in the power station and remaining Antipodean strains.

After these original studies were completed, isolates of *N. fowleri* from an electricity power station in France and the Moselle river supplying it were received (strains 3e7, 3e9, Na 420c and Na 1165b). These were also studied for their Bgl II and Bgl II-pB2.2 RFLPs. In addition, a strain of *N. fowleri* from a case of PAM in Hong Kong (HK-1) and an environmental isolate from Czechoslovakia (NF-59) were also compared. Of the four French strains, 3e7 and 3e9 gave agarose gel RFLPs identical to *N. fowleri* from the Bath hot springs, Belgium and the USA, as did the isolates from Hong Kong and Czechoslovakia Figure 74, Figure 76. Strains Na 420c and Na 1165b were identical to the strains from the Nottingham power stations and also the Antipodes. Hybridisation with pB2.2 gave RFLPs for 3e7 and 3e9 in common with KUL (Belgium), NF-59 (Czechoslovakia), HK-1 (Hong Kong), NF-124 (USA), Ng 060 (Australia) and MSM (New Zealand). Strains Na 420c and Na 1165b were identical to the Nottingham power stations isolates and showed the unique ~2.4 kbp RFLP absent from all other *N. fowleri* (Figure 77).

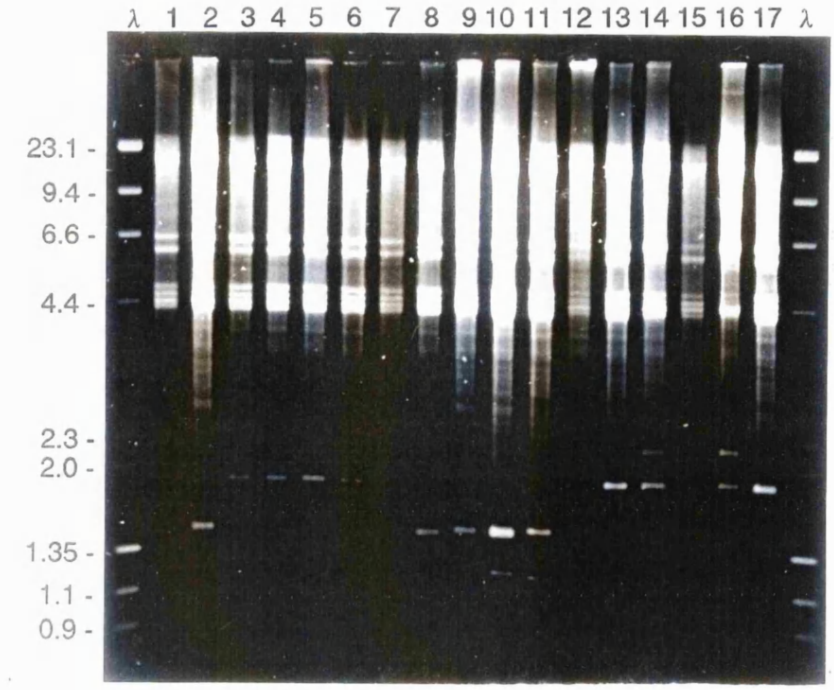

Figure 74 *N. fowleri* Bgl II RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) MCM, (2) NF-3, (3) A-44-1, (4) 1-44-2, (5) 152-44-3, (6) 162-44-5, (7) 162-44-5, (8) KUL, (9) HB-1, (10) CDC:0487:1, (11) NF-124, (12) Carter 69, (13) 1518/3, (14) 1518/4, (15) Ng 060, (16) MSM, (17) NHI

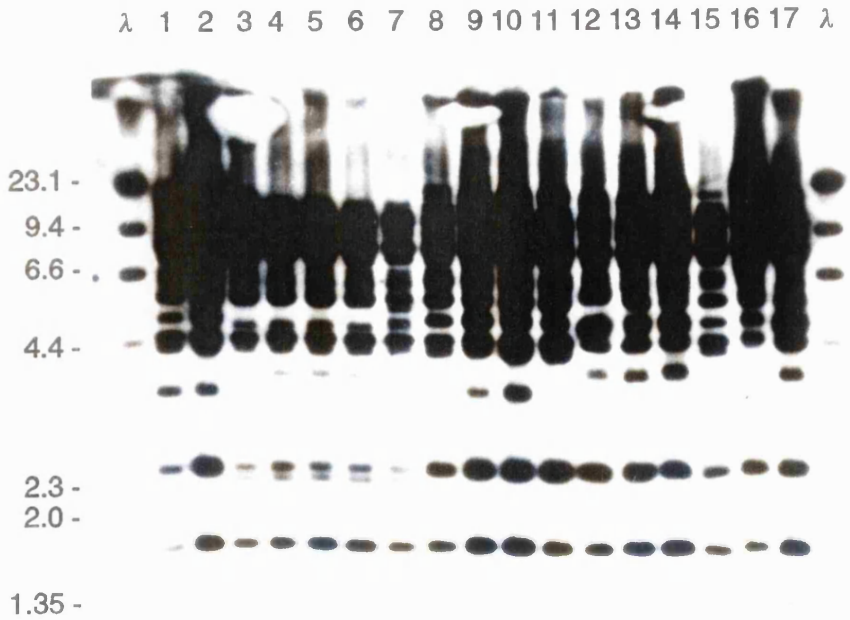

Figure 75 *N. fowleri* Bgl II-pB2.2 RFLPs

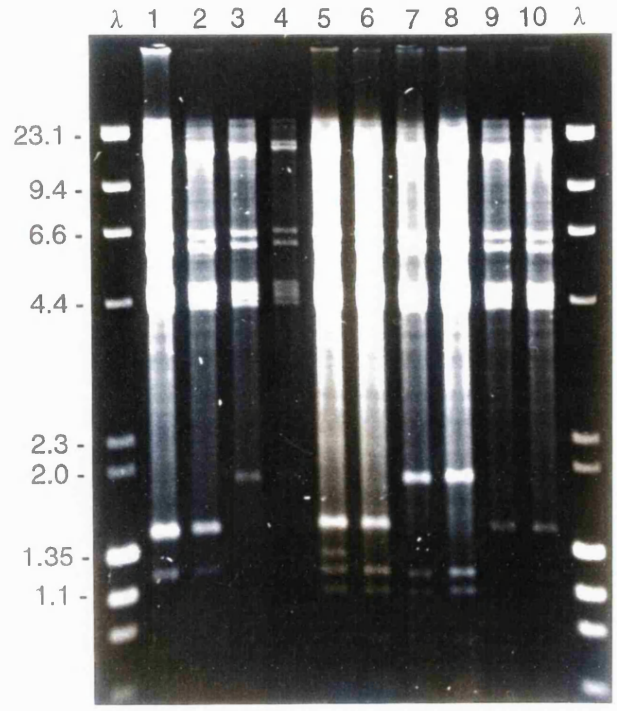

Figure 76 Additional *N. fowleri* Bgl II RFLPs

(λ) lambda-Hind III/ΦX-174 RF-Hae III digest. *N. fowleri*: (1) NF-3, (2) Q7089-1, (3) 1-44-2, (4) A-44-1, (5) 3e9, (6) 3e7, (7) Na 420c, (8) Na 1165b, (9) NF-59, (10) HK-1

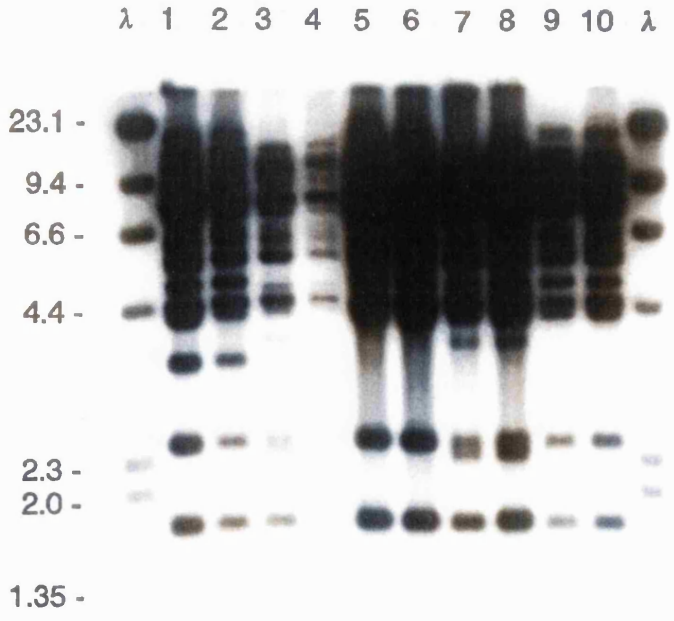

Figure 77 Additional *N. fowleri* Bgl II-pB2.2 RFLPs

The Bgl II and Bgl II-pB2.2 RFLPs for all the *N. fowleri* strains were compared for the presence or absence (1 or 0 state) of restriction fragments (and hence RFLPs). The numeric profiles or "operational taxonomic units" (OTU's) obtained were then analyzed using the Jaccard coefficient to construct a similarity matrix. This was then analyzed by the UMPGA programme of the Phylogeny Inference Package Version 3.2 (PHYLIP) of Joseph Felsenstein (1989). The output data was then used to produce the phenograms shown in Figure 78, Figure 79 and Figure 80.

With the Bgl II agarose gel RFLPs, two distinct groups (I and II) were obtained. Group II comprised the Antipodean, Nottingham and French power station (Na 420c and Na 1165b) strains and group I the remainder (Figure 78). With the pB2.2 probe RFLPs, subdivision of these two groups occurred (Figure 79). Within group I, strains from the Bath hot spring, HB-1 and CDC-0487-1 from the USA formed the subgroup IA, and the remainder subgroup IB. This subgroup now also included strains Ng 060 and MSM from Australia and New Zealand. Within group II, the Nottingham and French power station strains (Na 420c and Na 1165b) formed a subgroup IIA, separate from the remaining Antipodean strains assigned to subgroup IIB. When the Bgl II and Bgl II-pB2.2 RFLPs were analyzed together, the same subgroups were maintained except that Ng 060 and MSM now formed a separate subgroup IIC within the Antipodean-power station complex of group II (Figure 80).

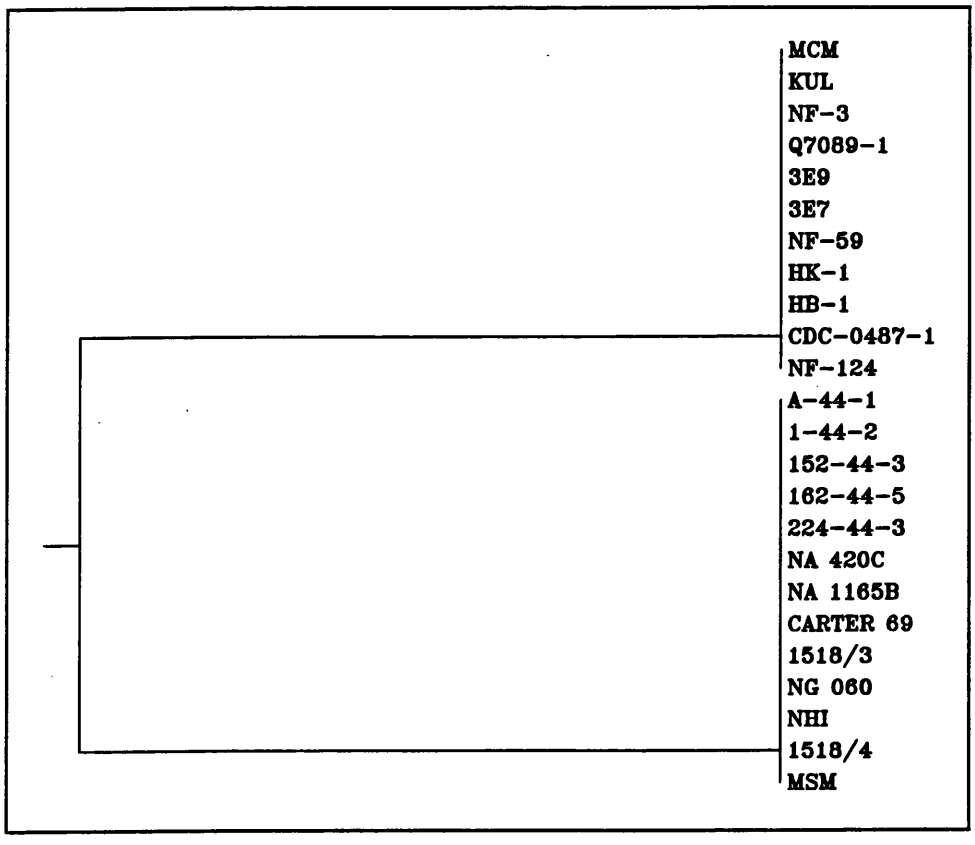

Figure 78 UMPGA analysis of *N. fowleri* Bgl II agarose gel RFLPs

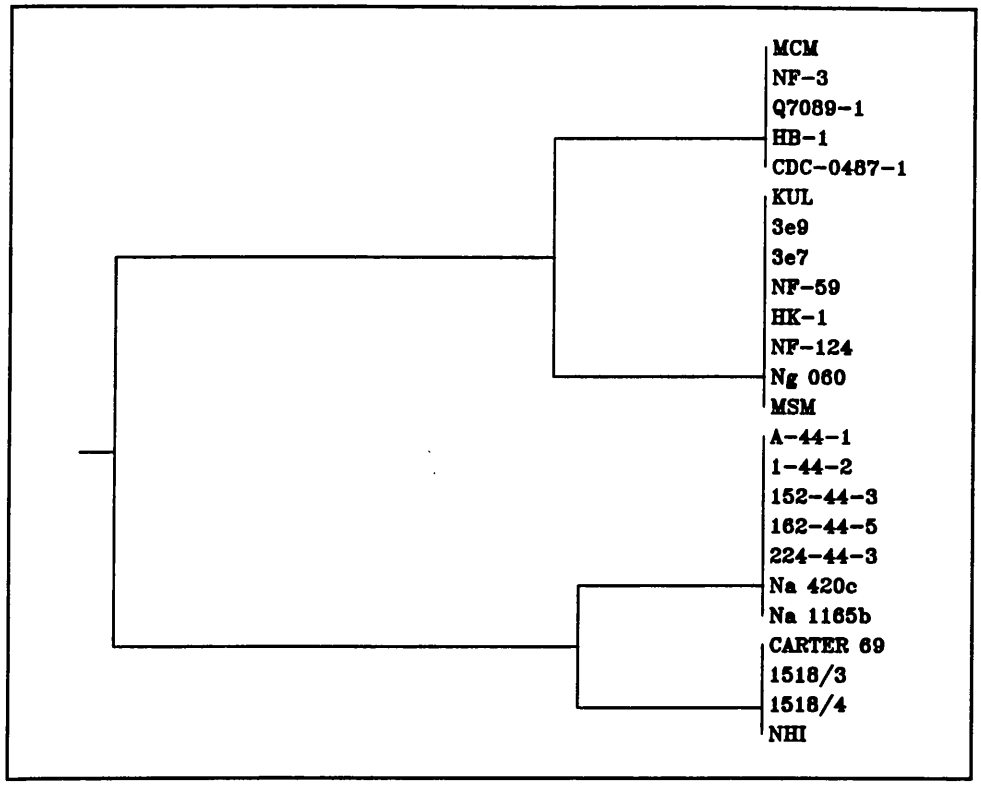

Figure 79 UMPGA analysis of *N. fowleri* Bgl II-pB2.2 probe RFLPs

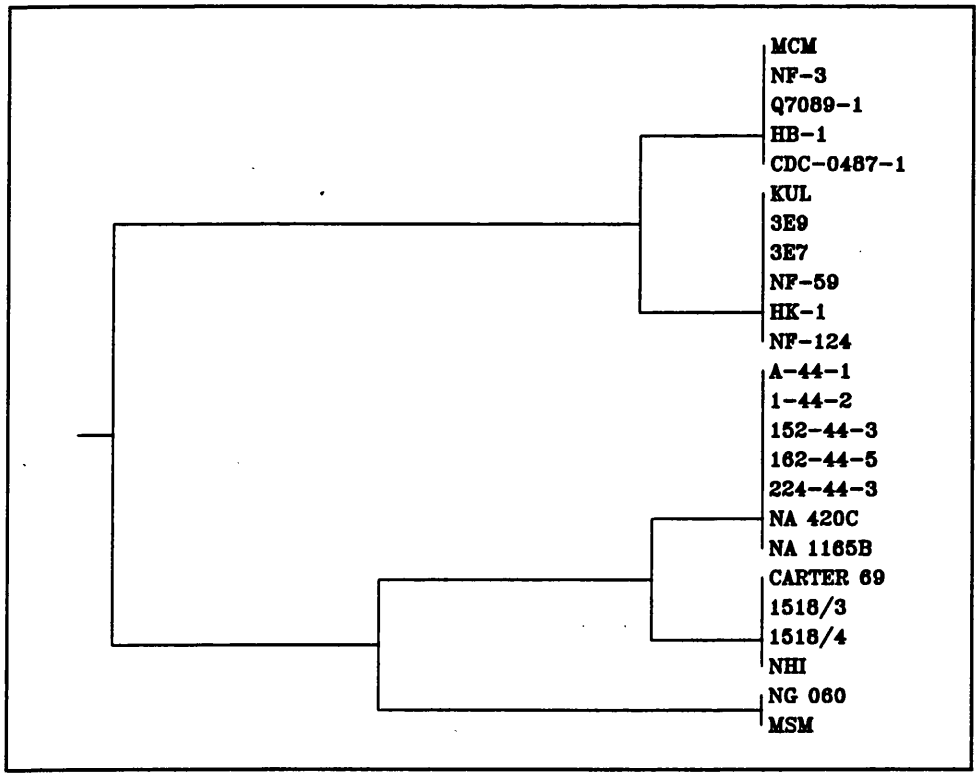

Figure 80 UMPGA analysis of *N. fowleri* Bgl II agarose gel and Bgl II-pB2.2 probe RFLPs

The groups to which the *N. fowleri* strains could be assigned on the basis of their Bgl II and pB2.2 RFLPs are summarised in Table VIII.

Table VIII. Differentiation of *N. fowleri* strains by Bgl II agarose gel and DNA probe RFLPs

		RFLP GROUP ASSIGNMENT		
Strain	Origin	Bgl II	pB2.2	Bgl II-pB2.2
MCM	Bath, England	I	IA	IA
NF-3	"	I	IA	IA
Q7089-1	"	I	IA	IA
HB-1	USA	I	IA	IA
CDC:0487:1	"	I	IA	IA
KUL	Belgium	I	IB	IB
3e9	France	I	IB	IB
3e7	France	I	IB	IB
NF-59	Czechoslovakia	I	IB	IB
NF-124	USA	I	IB	IB
HK-1	Hong Kong	I	IB	IB
1-44-1	Nottingham, England	II	IIA	IIA
1-44-2	"	II	IIA	IIA
152-44-3	"	II	IIA	IIA
162-44-5	"	II	IIA	IIA
224-44-3	"	II	IIA	IIA
Na 420C	France	II	IIA	IIA
Na 1165b	"	II	IIA	IIA
Carter 69	Australia	II	IIB	IIB
1518/3	"	II	IIB	IIB
1518/4	"	II	IIB	IIB
NHI	New Zealand	II	IIB	IIB
MSM	"	II	IB	IIC
Ng 060	Australia	II	IB	IIC

7.5. Discussion

The detection of RFLPs directly on agarose gels has been shown previously to be a powerful technique for the identification of *N. fowleri* and the detection of inter-strain differences which appeared to correlate with their geographic origin (De Jonckheere, 1987c & 1988b). Strains from Europe were found to be different from those of the Antipodes, whilst within the USA either profile occurred. Recently however, some strains of *N. fowleri* from an electricity power station in France and the Moselle river associated with the site (Dive *et al*, 1982) have been shown to display the Antipodean RFLP type previously unreported in Europe (Pernin & De Jonckheere, 1992). In the study reported here, *N. fowleri* isolates from two power stations in Nottingham, England and the River Trent supplying them were also identical to strains from the Antipodes in their agarose gel RFLPs. However, hybridisation of Bgl II digests with the *N. fowleri* repeated element DNA probe enabled these strains, and those from the French power station, to be differentiated from those of the Antipodes by the presence of a unique ~2.4 kbp RFLP. The Bgl II RFLPs obtained by the combination of these two methods enabled *N. fowleri* to be assigned to subgroups IA, IB, IIA, IIB or IIC. Of these, IA and IB comprised strains from Europe and the USA, IIA the English and French power stations, IIB the Antipodes, and IIC one strain each from Australia and New Zealand.

It has been speculated that because *N. fowleri* in the USA displays either European or Antipodean RFLP type that this is the most probable origin from which the organism has colonised other continents (De Jonckheere, 1988b). The identification of *N. fowleri* strains in France displaying the Antipodean RFLP type was attributed to recent introduction from outside of Europe, perhaps by human intervention (Pernin & De Jonckheere, 1992). However, the finding here that the English and French power station isolates were distinct from those of the Antipodes indicates that these *N. fowleri* strains in Europe has evolved separately. Furthermore, whilst *N. fowleri* from Japan have been shown to be similar to those from the Antipodes (De Jonckheere, *et al*, 1992), the strain from Hong Kong studied here was found to be of the Bgl II subgroup IB found only in strains from Europe and the USA. The origins of the geographic dissemination of *N. fowleri* are therefore unclear. Although only the Antipodean RFLP type (Bgl II subgroups IIB and IIC) has ever been found in Australia and New Zealand (De Jonckheere, 1987c & 1988b), the presence of the European type cannot be excluded as only a limited number of strains have been examined. In the study of the French power station isolates, both the European and Antipodean RFLP types (Bgl II subgroups IB and IIA) were identified (Pernin & De Jonckheere, 1992). Isolates made before 1987 were all found to be of European RFLP type, whilst those from 1987 to 1988 were of the Antipodean profile. Examination of four further isolates made in 1991 showed only one to be of Antipodean RFLP type. Previously, the only other isolates of *N. fowleri* from the United Kingdom have come from a fatal case of PAM in Bath, England and the local hot springs which were the source of the infection (Cain *et al*, 1981; Kilvington *et al*, 1991). Of some twenty isolates made over several years from this site, all have been found to be of the European RFLP type of Bgl II subgroup IA which has never been found in twelve of the English power station isolates made over two years all of which were Bgl II RFLP subgroup IIA. Perhaps *N. fowleri* of the various RFLP subgroups occurs in all continents but that in a given locality or

region only one type tends to predominate. Further studies using different restriction endonucleases and additional strains of *N. fowleri* from diverse geographic localities may enable the more specific differentiation of the organism for the purposes of epidemiological subtyping.

In conclusion, the detection of RFLPs directly on agarose gel electrophoresis and in combination with the repeated element chromosomal DNA probe is a powerful approach to the differentiation of *N. fowleri* strains and enabled the identification of a unique Bgl II subgroup of *N. fowleri* so far found only in power stations in France and England. As this RFLP subgroup has not been detected at the Bath hot springs, this indicates that the two sites have been colonised from separate sources and further supports the conclusion that *N. fowleri* is more widely distributed in this country than has previously been supposed as discussed in section *5. The isolation of Naegleria fowleri from water cooling towers associated with electricity production in Nottingham, England.*

Table IX. *N. fowleri* strains examined from the Nottingham power stations and other geographic localities

Species	Strain	Origin	Source
N. fowleri	MCM	Bath, England (PAM)	a
	Q7089-1	Thermal spa water, Bath, England	a
	NF-3	Thermal spa water, Bath, England	a
	A-44-1	Electricity power station, England	a
	1-44-2	"	a
	152-44-3	River water, England	a
	162-44-5	River water, England	a
	224-44-3	Electricity power station, England	a
	KUL	Belgium (PAM)	b
	3e9	Electricity power station, France	c
	3e7	Electricity power station, France	c
	Na 420c	Moselle river, France	c
	Na 1165b	Moselle river, France	c
	NF-59	Czechoslovakia	d
	HB-1	USA (PAM)	b
	CDC:0487:1	USA (PAM)	e
	NF-124	Thermal water, USA	b
	Carter 69	Australia (PAM)	b
	CCAP 1518/3 (Morgan)	Australia (PAM)	f
	CCAP 1518/4 (PA-90)	Domestic water supply, Australia	f
	Ng 060	Domestic water supply, Australia	g
	MSM	New Zealand (PAM)	f
	NHI	New Zealand (PAM)	b
	HK-1	Hong Kong (PAM)	h

PAM = from a case of primary amoebic meningoencephalitis

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8. CONCLUSIONS

The primary objective of this project was to develop improved methods for the rapid and sensitive detection of *N. fowleri* from the environment. To this end, cellulose acetate membrane electrophoresis (CAME) of glucose phosphate isomerase (GPI), DNA probes and the polymerase chain reaction (PCR) were applied successfully to the identification of the organism from the thermal springs complex in Bath and also electricity power stations and the River Trent in Nottingham, England. Of these methods, CAME of GPI is, perhaps, the simplest to perform as it requires little specialised equipment or particular expertise and has the advantage of enabling the identification of other thermophilic *Naegleria* spp. However, the test is limited by the need to subculture isolates following primary isolation and also the number of strains that can be examined on each membrane. This is an important consideration as samples from natural thermal aquatic sites can yield large numbers of thermophilic *Naegleria*, making the isoenzyme testing of isolates a time consuming and expensive process.

In contrast, the development of DNA probe technology enabled the identification of *N. fowleri* from the thermal springs directly from primary culture isolation plates. This process was facilitated by the use of simple method for the preparation of DNA from strains by direct lysis in NaOH in microtitre plates and immobilisation on to nylon membranes using a dot-blotting apparatus for subsequent hybridisation studies. The numbers of *N. fowleri* identified were greater than in past surveys when isoenzyme or whole-cell DNA restriction fragment length polymorphisms (RFLPs) were used. This is due, in part, to the fact that previously only randomly selected isolates were processed for identification because of the practical constraints of the detection methods. However, another important consideration is that the nonpathogenic thermophile *N. lovaniensis* grows at a faster rate than *N. fowleri* on primary culture isolation media and may suppress the presence of the latter. Accordingly, the advantage of the DNA probe strategy developed here is that all isolates to be readily sampled as soon as they emerge from the inocula on the culture plates and before possible overgrowth by *N. lovaniensis* and other thermophilic FLA can occur. A disadvantage is the labelling of the probes with 5'-[α-^{32}P] deoxycytidine triphosphate. Whilst this enables sensitive detection of hybridisation, such probes must be prepared fresh each time because of the short half-life of the isotope and cannot be reused. Several nonradioactive methods for the labelling of DNA are now available commercially, most commonly using a biotin or digoxigenin molecule coupled to a nucleotide in the DNA labelling reaction. Typically, probes labelled by such methods are stable for many months, can be reused several times and the detection of hybridisation is usually complete within hours rather than days. The use of such labelling methods in the development of nonradioactive DNA probes for the detection of *N. fowleri* warrants investigation.

Perhaps the most significant improvement in methodology for the identification of *N. fowleri* has been the development of the PCR for the organism. As with the DNA probes, a simple and rapid method was developed for the extraction of DNA from the amoebae for PCR using the Taq polymerase reaction buffer and proteinase K. Once the proteinase K had been inactivated by heating, the PCR could be performed

directly in the same tube. This protocol permited the sensitive and early detection of the organism from environmental samples as soon as trophozoites appeared on the primary isolation plates. Unlike the DNA probes, no radioisotopes or autoradiography are involved and the PCR and agarose gel electrophoresis detection of amplified product (and hence identification of *N. fowleri*) can be completed within one day. For primer sets pB2.3, a 1.5 kbp PCR product is produced which is of sufficient size to be unambiguously identified on agarose gel electrophoresis. A potential disadvantage of the PCR technique is its extreme sensitivity which, in theory, can result in amplification of a a single DNA molecule. Therefore, the opportunity for contamination leading to false positive results is substantial. Negative controls using samples lacking any DNA should be included in every assay and where environmental isolates are being identified, a known strain of *N. lovaniensis* should also be included. With both the DNA probe and PCR strategies developed here for the detection of *N. fowleri* from the environment, the isolates are also recovered into the wells of microtitre plates containing NNA-*E. coli* or NNA-K. *edwardsii*. This enables strains identified as *N. fowleri* by either of these methods to be verified by mouse pathogenicity, isoenzyme or whole-cell DNA RFLP analysis.

As with the DNA probes, the PCR only identifies *N. fowleri*. However, it is often important to be able to accurately identify *N. lovaniensis* as the presence of this amoebae in an environment is an indicator that conditions are suitable for the presence of *N. fowleri*. The development of the PCR for *N. lovaniensis* would also be advantageous. If the primers were so designed that the amplification product was significantly different in size to that of *N. fowleri*, then it may be possible to perform the PCR using primer sets to both species for identification. The application of the PCR to the detection of *N. fowleri* in environmental samples without the need for primary culture isolation may also be worth investigating. However, the possibility of false positive reactions with the test stresses the importance of being able to refer back to an actual culture isolate for further verification.

Prior to this study, the only isolates of *N. fowleri* in the United Kingdom have come from a PAM case in Bath and the thermal springs in the City which were the source of the infection. The isolation of *N. fowleri* from the power stations and River Trent in Nottingham are of important significance as they indicate that the organism is more widely distributed in manmade thermal aquatic sites and rivers in this country that has previously been supposed. RFLPs of the strains, detected by a combination of agarose gel and a DNA probe containing a repeated element clone of *N. fowleri* chromosomal DNA, showed them to be distinct from those found in the thermal springs of Bath. This suggests that these two sites have been colonised independently of one another. There is a need to investigate other thermal aquatic sites in this country to assess the distribution of *N. fowleri*. PAM is almost invariably fatal but is preventable if sites containing the organism can be identified and preventative public health measures implemented. In such surveys the ability to rapidly and reliably identify *N. fowleri* using the molecular techniques developed here would prove invaluable.

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APPENDIX 1. : CULTURE MEDIA

1.1. NON-NUTRIENT AGAR *E. COLI* MEDIUM (NNA-*E. COLI*)[1]

¼ strength Ringer's solution . . . . . 1000 ml
Plain agar . . . . . 15 g

Autoclave at 121°C for 15 minutes.
Dry plates over night at 37°C and store for up to 1 month at 4°C in sealed polythene bag.

Preparation of *E. coli*

Grow *E. coli* (JM101 or any strain will do) on blood agar plate over-night at 37°C. Store plate at 4°C in sealed polythene bags. Sub-culture weekly.

Preparation of NNA-*E. coli* plates

Scrape a generous portion of *E. coli* from culture plate using a cotton-tipped swab moistened with ¼ strength Ringer's solution.

Spread *E. coli* evenly over the surface of an NNA plate.

If several plates are to be seeded, scrape all the *E. coli* from a blood agar plate and resuspend in 3 ml of ¼ strength Ringer's solution. Inoculate 3 drops of suspension per NNA plate and spread with a sterile swab.

Store NNA-*E. coli* plates for up to 1 week at 4°C in sealed polythene bags.

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[1] *Klebsiella edwardsii* strain K10896 may also be used as a bacterial food source for the monoxenic culture of FLA

1.2. MODIFIED SERUM-CASEIN-GLUCOSE-YEAST EXTRACT MEDIUM (#SCGYM)[2]

#1.

Casein digest	10 g
Yeast extract	5 g
Glucose	2.5 g
Na_2HPO_4	1.325 g
KH_2PO_4	0.8 g
dH_2O	to 890 ml

Adjust to pH 6.9 - 7.0 if necessary and autoclave at 121°C for 15 minutes. Store at 4°C.

#2.

Panmede liver digest	10 g
dH_2O	to 100 ml

Adjust to pH 6.9 with 1 M NaOH and filter sterilise. Distribute in 10 ml volumes and store frozen at -20°C.

For use:

#1	890 ml
#2	10 ml
Foetal calf serum (heat inactivated)	100 ml
Penicillin (10,000 U /ml)	2.5 ml
Streptomycin (10,000 U /ml)	2.5 ml

Store complete medium at 4°C

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[2] Chang, S.L. (1974). Cytopathic and pathogenic differences among geographic strains of pathogenic *Naegleria* and their bearing on epidemiology of primary amoebic meningoencephalitis. *3rd International Congress of Parasitology, (Munich)*. **1**: 187-188.

Modified by the inclusion of 0.1% Panmede liver digest

1.3. MODIFIED YEAST EXTRACT-PEPTONE-YEAST NUCLEIC ACID-FOLIC ACID-HAEMIN CULTURE MEDIUM (#YPNFH)[3]

#1.	Difco peptone Nº3	10.0 g
	Difco yeast extract	10.0 g
	Yeast nucleic acid (Sigma: store at -20°c)	1.0 g
	D-glucose	1.0 g
	Folic acid	15 mg
	*Haemin	0.5 ml
	KH_2PO_4	0.362 g
	Na_2HPO_4	0.5 g
	dH_2O	to 900 ml

Adjust to pH 6.9 and autoclave at 121°C for 15 minutes. Store at 4°C.

***Haemin solution**

Haemin	200 mg
Triethanolamine (50% in dH_2O)	100 ml

Store at 4°C.

For use:

#1	900 ml
Foetal calf serum (heat inactivated)	100 ml
Penicillin (10,000 U /ml)	10 ml
Streptomycin (10,000 U /ml)	10 ml

Store at 4°C and use within 1 month.

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[3] Laverde, A.V. and Brent, M.M. 1980. Simplified soluble media for the axenic cultivation of *Naegleria*. *Protistologica* XVI: 11-15.

Modified by the inclusion of glucose, phosphate salts and 10% foetal calf serum.

1.4. NZY BROTH

NaCl	5.0 g
$MgSO_4 .7H_2O$	2.0 g
Yeast Extract	5.0 g
NZ Amine	10.0 g
dH_2O	to 1000 ml

Adjust to pH 7.5 and autoclave at 121°C for 15 minutes.

1.4.1. NZY AGAR

NZY broth	1000 ml
Purified agar or agarose	15.0 g

1.4.2. NZY TOP AGAR (0.8%)

NZY broth	100 ml
Agarose	0.8 g

Autoclave at 121°C for 15 minutes.

1.5. TB MEDIA WITH $MgSO_4$ AND MALTOSE

NaCl	5.0 g
Bacto tryptone	10.0 g
dH_2O	to 980 ml

Adjust to pH 7.4 and autoclave at 121°C for 15 minutes.

Cool and add the following filter sterilised supplements:

$MgSO_4$ (1 M)	10 ml (10 mM)
Maltose (20%)	10 ml (0.2%)

1.6. λ SM BUFFER

NaCl	5.8 g
$MgSO_4 .7H_2O$	2.0 g
Tris-HCl (1M, pH 7.5)	50 ml
Gelatin	20 g
dH_2O	to 1000 ml

Autoclave at 121°C for 15 minutes.

1.7. λ PHAGE DILUTION BUFFER

Tris-HCl (pH 8.0)	10 mM
$MgSO_4$	10 mM
EDTA	1 mM

Autoclave at 121°C for 15 minutes.

1.8. $MgSO_4$ (10 mM)

$MgSO_4$,1 M (filter sterilised)	10 ml
dH_2O (sterile)	90 ml

1.9. 20% PEG 8000:12% NaCl

Polyethylene glycol	100 g
NaCl	60 g
dH_2O	to 500 ml

1.10. LURIA-BERTANI (LB) BROTH

Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
dH_2O	1 L

Adjust to pH 7.0 with 1 N NaOH and autoclave at 12lbs pressure for 15 minutes.

1.11. LURIA-BERTANI (LB) AGAR

LB broth . . . . . 1000 ml
Agar . . . . . 15 g
Adjust to pH 7.0 with 1 N NaOH and autoclave at 12lbs pressure for 15 minutes.

1.12. TERRIFIC BROTH

Bacto-tryptone . . . . . 12 g
Bacto-yeast extract . . . . . 24 g
Glycerol . . . . . 4 ml

Dissolve in 900 ml of dH_2O with warming and autoclave at 121°C for 15 minutes.

Cool to 60°C or less and add 100 ml of:

KH_2PO_4 . . . . . 0.17 M
K_2HPO_4 . . . . . 0.72 M

This is prepared by dissolving 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 ml of dH_2O. When dissolved make up to 100 ml and autoclave at 121°C for 15 minutes.

APPENDIX 2. : MOLECULAR BIOLOGY REAGENTS

2.1. GLUCOSE PHOSPHATE ISOMERASE (GPI) CELLULOSE ACETATE MEMBRANE ELECTROPHORESIS BUFFER

Tank buffer

Tris base	12.11 g
EDTA(Na_2)	33.62 g
$MgCl_2$.$6H_2O$	20.33 g
Maleic acid	11.61 g
dH_2O	to 1 L

Dissolve the chemicals in 800 ml of dH_2O in the order listed above ensuring each has fully dissolved before adding the next. The disodium EDTA will not dissolve in acidic solution. Adjust to pH 7.4 with 10 N NaOH (~30 ml) and make up to 1 L with dH_2O.

Membrane buffer

This is a 1:15 dilution of the tank buffer in dH_2O. Check the pH is 7.4.

2.1.1. GPI DEVELOPING SOLUTION

Tris-HCl (0.1 M, pH 8.0)	100 ml
EDTA(Na_2)	100 mg
$MgCl_2$.$6H_2O$	200 mg
Fructose-6-phosphate (Sigma, F 3627)	35 mg
ß-NADP (Sigma, N 0505)	25 mg
Glucose-6-phosphate dehydrogenase (Sigma, G7877)	40 units

Distribute in 1 ml aliquots and store frozen at -20°C or -70°C.

For use, thaw one aliquot and add:

MTT (Sigma, M 2128)	0.75 mg
Phenazine methasulphate (Sigma, P 9625)	0.45 mg

These are delivered as "knife-point" quantities. Use immediately and protect from light.

2.2. DNA CELL LYSIS BUFFER

Tris-HCl (pH 8.0)	10 mM
NaCl. . .	100 mM
EDTA (Na_2)	100 mM

Autoclave at 121°C for 15 minutes and store at room temperature.

2.3. PROTEINASE K

Dissolve at 10 mg/ml in dH_2O. Incubate at 37°C for 1 hour. Aliquot and store at -20°C.

2.4. N-LAUROYLSARCOSINE (SARKOSYL: 20%)

Dissolve 20 g of N-laurylsarcosine (sodium salt) in dH_2O to give a final volume of 100 ml. Adjust to pH 7.0 if necessary. Autoclave at 121°C for 15 minutes and store at room temperature.

2.5. SODIUM DODECYL SULPHATE (SDS: 10%)

Dissolve 10 g of sodium dodecyl sulphate in dH_2O to give a final volume of 100 ml. Adjust to pH 7.0 if necessary. Autoclave at 121°C for 15 minutes and store at room temperature.

2.6. PANCREATIC RIBONUCLEASE A

Dissolve RNase at 5 mg/ml in 10 mM Tris-HCl, pH 7.5. Place in boiling water bath for 10 minutes and cool slowly at room temperature. Aliquot and store at -20°C.

2.7. 3 M SODIUM ACETATE, pH 5.2

Sodium acetate	40.8 g
dH_2O	80 ml

Titrate to pH 5.2 with glacial acetic acid and make up to 100 ml with dH_2O.
Autoclave at 121°C for 15 minutes and store at room temperature.

2.8. TE BUFFER*

Tris-HCl, pH 7.5 . . . . . 10 mM
EDTA (Na_2) . . . . . 1 mM

Autoclave at 121°C for 15 minutes and store at room temperature.

*TE buffer at pH 8.0 is prepared using 10 mM Tris-HCl at pH 8.0.

2.9. PHENOL:CHLOROFORM:ISOAMYL ALCOHOL (25:24:1)

Phenol . . . . . 100 g
Chloroform . . . . . 96 ml
Isoamyl alcohol . . . . . 4 ml
8-hydroxyquinoline . . . . . 100 mg

Dissolve the phenol crystals in chloroform and add isoamyl alcohol and 8-hydroxyquinoline.

Extract twice with 200 ml of 1 M tris-HCl, pH 8.0 and once with 0.1 M tris-HCl, pH 8.0. Aliquot in light-proof glass bottles and overlay with 30 ml of 0.1 M tris-HCl, pH 8.0. Store frozen at -20°C.

2.10. CHLOROFORM:ISOAMYL ALCOHOL (24:1)

Chloroform . . . . . 96 ml
Isoamyl alcohol . . . . . 4 ml

Overlay with dH_2O and store in light-proof glass bottle at 4°C.

2.11. 10X TRIS-BORATE-EDTA (TBE) ELECTROPHORESIS BUFFER

Tris base (108 g/ L) . . . . . 0.89 M
Boric acid (54 g/ L) . . . . . 0.89 M
EDTA([Na_2] 9.2 g/ L) . . . . . 0.025 M

Adjust to pH 8.2-8.3 if necessary. Use at 0.5X concentration for agarose gel electrophoresis.

2.12. 10X TRIS-ACETATE EDTA (TAE) ELECTROPHORESIS BUFFER

Tris base	48.4 g
Glacial acetic acid	11.4 ml
EDTA (0.5 M, pH 8.0)	20 ml
dH_2O	to 1 L

Adjust to pH 7.9 if necessary. Autoclave at 121°C for 15 minutes. Use at 1X concentration for agarose gel electrophoresis.

2.13. ETHIDIUM BROMIDE

Dissolve ethidium bromide (EBr) at a concentration of 10 mg/ml in dH_2O overnight using a magnetic stirrer. Wrap the bottle in aluminium foil and store in the dark at 4°C.

Add 50 $\mu\ell$ EBr /L of TBE buffer and 5 $\mu\ell$ EBr /100 ml of agarose gel (final concentrations of 0.5 μg/ml) or 100 $\mu\ell$ /L of dH_2O if agarose gel is stained after electrophoresis.

- CAUTION, ETHIDIUM BROMIDE IS A POWERFUL MUTAGEN -

2.14. GEL SAMPLE LOADING BUFFER AND RESTRICTION ENZYME STOP MIXTURE (10X)

Sucrose	20%
Ficoll	10%
EDTA (Na_2), pH 8.0	0.1 M
Bromophenol blue	1%

2.15. 20X SSC

NaCl (175.3 g /L)	3 M
Trisodium citrate (88.2 g /L)	0.3 M

Adjust to pH 7.0 with 0.1 N NaOH and sterilise by autoclaving at 121°C for 15 minutes.

2.16. ALKALINE DNA TRANSFER SOLUTION

NaOH . . . . . 0.5 M
NaCl . . . . . 1.5 M

2.17. ALKALINE DNA TRANSFER NEUTRALISING SOLUTION

Tris HCl (pH 7.2) . . . . . 0.5 M
NaCl . . . . . 1.5 M
EDTA . . . . . 1 mM

2.18. GLUCOSE-TRIS-EDTA (GTE)

Tris-HCl (pH 8.0) . . . . . 25 mM
EDTA (pH 8.0) . . . . . 10 mM
Glucose . . . . . 50 mM

Autoclave at 121°C for 15 minutes and store at 4°C.

2.19. 3 M POTASSIUM ACETATE

Potassium acetate (5 M) . . . . . 60 ml
Glacial acetic acid . . . . . 11.5 ml
dH_2O . . . . . 28.5 ml

Autoclave at 121°C for 15 minutes.The solution is 3 M with respect to potassium and 5 M with respect to acetate.

2.20. SPERMIDINE TRIHYDROCHLORIDE (100 mM)

Dissolve 2.556 g in dH_2O and make up to 100 ml. Filter sterilise, aliquot and store at -20°C.

2.21. 10X CALF INTESTINAL ALKALINE PHOSPHATASE BUFFER

Tris-HCl (pH 9.0) . . . . . 500 mM
$MgCl_2$ . . . . . 10 mM
$ZnCl_2$ . . . . . 1 mM
Spermidine trihydrochloride . . . . . 10 mM

2.22. 10X λ PHAGE LIGATION BUFFER

Tris-HCl, pH 8.0	500 mM
$MgCL_2$	100 mM
DTT	10 mM
ATP (100 mM, pH 7.5)	10 mM
BSA	1 mg/ml

Filter sterilise, aliquot and store at -20 °C.

2.23. DNA OLIGOLABELLING BUFFER (OLB)

Each triphosphate is dissolved at a concentration of 0.1 M in 3 mM Tris-HCl, 0.2 mM EDTA(Na_2), pH 7.0.

A.

Tris-HCl (2 M), pH 8.0	625 $\mu\ell$
$MgCl_2$	25 $\mu\ell$
dH_2O	350 $\mu\ell$
2-mecaptoethanol	18 $\mu\ell$
dATP	5 $\mu\ell$
dTTP	5 $\mu\ell$
dGTP	5 $\mu\ell$

Store at -20°C

B. HEPES (2 M) adjusted to pH 6.0 with 1 M NaOH. Store at 4°C.

C. Hexadeoxyribonucleotides (Pharmacia 27-2166-01). Dissolve at a concentration of 90,000 units /ml in 3 mM Tris-HCl, 0.2 mM EDTA(Na_2), pH 7.0. Store at -20°C.

Mix **A**, **B** and **C** at a ratio of **2:5:3** to make OLB. Store at -20°C. This is stable for at least 3 months with repeated freezing and thawing.

2.24. DNA HYBRIDISATION SOLUTION

Add the following in order, allowing the BSA to fully dissolve (warm solution to 60°C) before adding the SDS:

Na_2HPO_4 (0.5 M, pH 7.2)	131.5 ml (0.263 M)
EDTA (0.5 M, pH 8.0)	0.5 ml (1 mM)
dH_2O	90 ml
Bovine Serum Albumin (fraction V)	2.5 g (1%)
SDS	17.5 g (7%)

Make up to 250 ml with dH_2O.

A 0.5 M stock solution of Na_2HPO_4 is:

Na_2HPO_4 (anhydrous)	71 g /500 ml

Adjust to pH 7.2 with ~3 ml of H_3PO_4 (85%)

2.25. SODIUM IODIDE SOLUTION (FOR SILICA DNA PURIFICATION)

Dissolve 90.8 g of NaI (sodium iodide) plus 1.5 g of Na_2SO_3 (sodium sulphite) in 100 ml of TE buffer (pH 7.4). Pass through a 0.45 μm filter. Add 0.5 g of Na_2SO_3. The solution should be saturated. Store in light proof bottle at 4 °C.

2.26. ETHANOL WASH SOLUTION (FOR SILICA DNA PURIFICATION)

NaCl	200 mM
Tris-HCl (pH 7.5)	20 mM
EDTA	2 mM
Ethanol (99%)	50%

2.27. 5X PLASMID LIGATION BUFFER

Tris-HCl, pH 7.6 (1 M stock. Autoclave) . . . . . 250 mM
MgCl2 (1 M stock. Autoclave) . . . . . 50 mM
PEG 8000 (50% stock. Filter sterilise) 25%
ATP (0.1 M stock, pH 7.0. Filter sterilise) . . . . . 5 mM
DTT (1 M stock in 0.01 M Na acetate, pH 5.2. Filter sterilise) . . . . . 50 mM

2.28. 2X *E. COLI* TRANSFORMATION AND STORAGE SOLUTION (2X TSS)

LB broth . . . . . to 90 ml
PEG 3350 (or 8000)* . . . . . 20 g (20%)
$MgSO_4$ . . . . . 40 mM

Adjust to pH 6.5-6.7 and filter sterilise. Distribute in 9 ml volumes and store at 4°C.

*Other molecular weight types of PEG are not suitable.

Immediately before use add to DMSO to 10%.

DMSO should be from a new unopened bottle. Aliquoted in small amounts and store at -70°C.

2.29. 10X TAQ POLYMERASE BUFFER

KCl . . . . . 500 mM
Tris-HCl (pH 9.0) . . . . . 100 mM
$MgCl_2$ . . . . . 15 mM
Triton X-100 . . . . . 1%

2.30. PCR-CELL LYSIS BUFFER

10X Taq polymerase buffer . . . . . 100 $\mu\ell$
Proteinase K (10 mg/ml) . . . . . 10 $\mu\ell$
dH_2O . . . . . 590 $\mu\ell$

Prepare fresh and distribute in 70 $\mu\ell$ volumes.

APPENDIX 3. : SCIENTIFIC METHODS

3.1. CELLULOSE ACETATE MEMBRANE ELECTROPHORESIS (CAME) OF GLUCOSE PHOSPHATE ISOMERASE (GPI)

1. Pellet trophozoites by centrifugation at 500 x g for 5 minutes at room temperature.

2. Resuspended in 1 ml of ¼ strength Ringer's solution and transfer to a 1.5 ml eppendorf tube

3. Centrifuged at 6,500 rpm for 1 minute at room temperature.

4. Remove all supernatant and resuspend the cell pellet in 50 $\mu\ell$ of dH_2O containing 0.2% Triton X-100 (v/v) and place on ice for 5 minutes with occasional mixing.

5. Vortex tube briefly and centrifuged at 13,500 rpm for 1 minute.

6. The supernatant is kept on ice and used immediately for were either used immediately for CAME. Supernatants can be stored at -20°C for at least 4 weeks.

7. Carefully immerse a cellulose acetate membrane in membrane buffer avoiding air bubbles and leave for 10 minutes.

8. Gently blot membrane between filter paper and apply samples. Usually, two sample applications are used for each lysate.

9. Electrophoresis is performed using a standard horizontal flat-bed electrophoresis tank with filter paper wicks for contact between the electrode buffer and membrane.

   Electrophoresis is conducted at a constant current of 12 mA for 15 minutes. GPI migration is from cathode (-) to anode (+).

10. Prepare GPI developing solution and pour on to a sheet of Saran® Wrap placed over a glass plate.

11. Position the membrane enzyme side down on to the developer avoiding air bubbles. Place a second glass plate was placed on top and fold the cellophane around the upper plate to prevent leakage of the developer.

12. Clamp the plates together and incubate in the dark at 37°C for 5-10 minutes.

13. Stop the reaction by submerging the membrane in 5% (v/v) acetic acid followed by washing in running tap water for 5 minutes.

14. The intensity of the bands fades with time. Therefore, the membranes are either photographed, or photocopied on to paper for a permanent record.

3.2. ISOLATION OF *NAEGLERIA* WHOLE-CELL DNA

1. Grow trophozoites to confluence in #SCGYM or #YPNFH medium in 1 x 180 cm^2 tissue culture flask.

2. Centrifuge at 2000 g for 10 minutes and wash cells once with phosphate buffered saline.

3. Suspend pelleted cells in 4 ml of cell lysis buffer.

4. Add 60 $\mu\ell$ of 10 mg/ml proteinase K (150 μg/ml) and mix.

5. Add 400 $\mu\ell$ of 20% Sarkosyl (2%) and mix gently.

6. Incubate at 56°C for 4 hours to overnight.

7. Pour into SST blood collection tube (Becton Dickinson).

8. Add 4 ml of phenol:chloroform:isoamyl alcohol (25:24:1). Gently mix for 5 minutes.

9. Centrifuge at 2000 g for 10 minutes.

10. Pour upper aqueous phase into fresh SST tube.

11. Extract once with chloroform:isoamyl alcohol (24:1) by centrifugation at 2000 g for 10 minutes.

12. Transfer upper aqueous phase to polypropylene centrifuge tube.

13. Add an equal volume of -20°C anhydrous iso-propanol. Mix gently and leave at -20°C for 1 hour to overnight.

14. Centrifuge at 2000 g for 20 minutes and remove all supernatant by aspiration.

15. Rinse pellet twice with 1 ml of 70% ethanol and once with absolute ethanol. Centrifuge briefly between each rinse and remove all supernatant with micropipette.

16. Dry pellet briefly in a 37°C incubator.

17. Dissolve pellet in 100 $\mu\ell$ TE buffer containing 25 μg /ml RNase A.

Pure DNA should have an absorbance ratio of $\geq$1.8 O.D. units at 260/280 nm. 1 O.D. unit at 260 nm is equal to 50 μg/ml of DNA.

3.3. RESTRICTION ENDONUCLEASE DIGESTION OF DNA

1X REACTION MIX

10X enzyme buffer . . . . . 20 $\mu\ell$
Spermidine trihydrochloride (100 mM) . . . . . 8 $\mu\ell$
Restriction endonuclease (8-12 U/$\mu\ell$) . . . . . 8 $\mu\ell$

Add 2 $\mu\ell$ to 18 $\mu\ell$ of DNA and incubate for 4 hours to overnight.

2X REACTION MIX

10X enzyme buffer . . . . . 40 $\mu\ell$
Spermidine trihydrochloride (100 mM) . . . . . 16 $\mu\ell$
Restriction endonuclease (8-12 U/$\mu\ell$) . . . . . 15 $\mu\ell$
dH_2O . . . . . 129 $\mu\ell$

Add to an equal volume of DNA and incubate for 4 hours to overnight.
There is sufficient reaction mix for 20 reactions if 10 $\mu\ell$ of DNA is used.

5X REACTION MIX

10X enzyme buffer	40 $\mu\ell$
Spermidine trihydrochloride (100 mM)	16 $\mu\ell$
Restriction endonuclease (~8-12 U/$\mu\ell$)	15 $\mu\ell$
dH_2O	9 $\mu\ell$

Add 4 $\mu\ell$ to 16 $\mu\ell$ of DNA and incubate for 4 hours to overnight.

There is sufficient reaction mix for 20 reactions.

3.4. AGAROSE GEL ELECTROPHORESIS OF NUCLEIC ACIDS

1. Dissolve agarose at required concentration in 0.5X TBE buffer or 1X TAE by boiling.[4]

2. Cool to 55°C and add ethidium bromide to 0.5 μg/ml if required.[5]

3. Seal ends of gel casting tray with autoclave tape and position well forming comb.

4. Pour agarose into the casting tray avoiding air-bubbles (gently flame molten gel to remove). Aim for a gel 0.5 cm thick.

5. Allow gel to set at room temperature and transfer to a refrigerator for 30 minutes.

6. Remove autoclave tape from ends of casting tray and carefully remove the well comb.

7. Leave the gel in the casting tray and place inside electrophoresis apparatus.

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[4] The fractionation range of DNA varies according to the following concentrations of agarose:

5-60 kbp	0.35%
2-20 kbp	0.7%
0.6-8 kbp	1%
0.5-6 kbp	1.2%
0.2-4 kbp	1.4%
0.1-2 kbp	2%

[5] The presence of ethidium bromide during electrophoresis reduces DNA migration by ~15% and can alter the relative order of mobility of plasmid DNA forms.

8. Pour 0.5X TBE buffer or 1X TAE buffer into the electrophoresis apparatus until the gel is submerged under a few millilitres of buffer.

9. Connect to apparatus to power supply and perform electrophoresis[6].

- DNA MIGRATION IS FROM CATHODE (-) TO ANODE (+) -

10a. If electrophoresis was conducted in the presence of ethidium bromide, carefully remove the casting tray with the gel and view gel under ultraviolet light at 305 nm.

10b. If electrophoresis was not conducted in the presence of ethidium bromide, stain the gel for 1 hour in the used electrophoresis buffer to which 1 μg/ml ethidium bromide and destain for 20 minutes in dH_2O before viewing under ultraviolet light.

11. Photograph gel using Polaroid 667 or 665 Instamatic film and a Polaroid MP-4 camera system. A Wratten 23A orange filter is placed between the gel and the camera and a Wratten 2A UV filter is also used between the light source and the 23A filter.

For Polaroid 667 film, typical exposure times with a lens aperture of 5.6 is a ½-1 second. The film is developed according to the manufacturer's instructions with a processing time of 40 seconds. Prints only are produced with this film.

For Polaroid 665 film, typical exposure times with a lens aperture of 4.5 is 45-60 seconds. The film is developed according to the manufacturer's instructions with a processing time of 40 seconds. Polaroid 665 film produces a medium quality print but good quality negative from which enlargements can be made.

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[6] 'Mini-gel' apparatus can be run at voltages of up to 14 v/cm for rapid analysis. However, better resolution is obtained with large gels electrophoresed at 1.5-2 v/cm overnight. For both methods, stop electrophoresis when the tracking dye has migrated ~75% along the length of the gel.

3.5. QUANTIFICATION OF DNA USING HOECHST H33258 DYE IN AGAROSE PLATES[7]

Under these conditions, the presence of RNA in the samples does not interfere with the quantification of the DNA

1. Prepare a 10 mg/ml stock solution of H33258 (bisbenzamide) in 10 mM Tris-HCl, pH 7.5. Store at 4°C in a light proof container.

2. Dissolve 1% agarose in dH_2O, cool to 55°C and add H33258 to 2.5 μg/ml (25 $\mu\ell$/100 ml).

3. Pour into petri dishes (20 ml/dish) and allow to solidify.

4. Spot 1 $\mu\ell$ volumes of standards (1, 0.5, 0.25, 0.125, 0.0625 $\mu g/\mu\ell$) and samples on to plates and allow to stand in the dark at room temperature for 60 minutes.

5. Remove petri dish lid and view under UV light at 305 nm wavelength.

6. Estimate DNA concentration of samples by comparing the fluorescence with that of the standards.

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[7] Rieber, M.S. and Rieber, M.A (1990). Hoechst H33258 agarose plate assay for the estimation of nanogram DNA levels without RNA interference: applications in PCR and in estimations of plasmid and cytoplasmic DNA. *Nucleic Acids Research*. **18**: 1918.

The presence of Ca^{2+} and Mg^{2+} quenches H33258 fluorescence.

3.6. PARTIAL DIGESTION OF GENOMIC DNA WITH RESTRICTION ENZYME SAU3A I

3.6.1. DEFINING THE ENZYME CONCENTRATION

1. DNA . . . . . 10 μg
   10X Sau3A I enzyme buffer . . . . . 15 $\mu\ell$
   Spermidine (100 mM) . . . . . 6 $\mu\ell$
   dH_2O to . . . . . 150 $\mu\ell$

2. Mix gently but thoroughly.

3. Add 30 $\mu\ell$ to tube 1 and 15 $\mu\ell$ to tubes 2-9.

4. Add 1 $\mu\ell$ of Sau3A I (5 U/$\mu\ell$) to tube 1. Mix well and transfer 15 $\mu\ell$ to tube 2. Continue to tube 9 and discard 15 $\mu\ell$.

5. Microfuge tubes for 2 sec and incubate at 37°C for 1 hour.

6. Place tubes on ice and add 1 $\mu\ell$ of 0.5 M EDTA (Na_2), pH 8.0.

7. Heat samples at 68°C for 5 minutes and place on ice.

8. Add 3 $\mu\ell$ of 10X stop-loading solution to all tubes and place on ice.

9. Perform electrophoresis in 0.5% gel at 2 v/cm overnight. Include appropriate size markers of λ Hind III. Stain with 1 μg/ml ethidium bromide in dH_2O for 30 minutes.

10. Photograph gel and record the enzyme concentration giving maximum fluorescence in the range 15-22 Kb.

TUBE Nº	1	2	3	4	5	6	7	8	9	10
Sau3A I (U)	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0.005

Each tube contains 1 μg of DNA in a total volume of 15 $\mu\ell$.

In large scale preps, **HALVE** the enzyme concentration to give the maximum number of molecules in this range.

PARTIAL DIGESTION OF GENOMIC DNA WITH RESTRICTION ENZYME SAU3A I (CONT)

3.6.2. LARGE SCALE DIGESTION

1. DNA . . . . . 200 μg
   10X Sau3A I enzyme buffer . . . . . 300 $\mu\ell$
   Spermidine (100 mM) . . . . . 120 $\mu\ell$
   dH_2O . . . . . to 3 ml

2. Warm at 37°C for 15 minutes.

3. Add appropriate amount of Sau3A I as determined from small scale preparations (**A**).

4. Incubate 37°C for 1 hour.

5. Immediately add:

   0.5 M EDTA (Na_2), pH 8.0 . . . . . 120 $\mu\ell$ (20 mM)

6. Heat at 68°C for 5 minutes and place on ice.

7. Phenol and Chloroform extract once.

8. Add 3 M Na Acetate to 1/10th of volume (312 $\mu\ell$) and 2.5 volumes of absolute ethanol. Place at -20°C overnight.

9. Centrifuge at 3200 rpm for 30 minutes.

10. Wash pellet twice with 70% ethanol and once with absolute ethanol.

11. Dry pellet briefly in a 37°C incubator.

12. Dissolve in 200 $\mu\ell$ of TE buffer.

13. Estimate DNA concentration using H33258 plate assay.

14. Ligate DNA and compare with unligated sample on a 0.5% agarose gel.

3.7. DEPHOSPHORYLATION OF DNA FOR CLONING INTO λ PHAGE EMBL3

Dephosphorylation with calf intestinal alkaline phosphatase (CIAP) is used to prevent self-ligation of restriction endonuclease digested DNA when cloning.

Note:

Dephosphorylation of DNA can be conveniently performed directly following restriction endonuclease digestion.

Controls:

Perform ligation reactions on phosphatase treated and untreated DNA and compare to unligated DNA on an agarose gel.

1. Restriction endonuclease digested DNA . . . . . 10 μg
   10X CIAP Buffer . . . . . 5 $\mu \ell$
   Calf intestine alkaline phosphatase (CIAP) . . . . . 5 Units
   dH_2O . . . . . to 50 $\mu \ell$

   Incubate at 37°C for 1 h.

2. Add 6 $\mu \ell$ of 100 mM trinitriloacetic acid (pH 8.0).

3. Heat at 70°C for 10 minutes.

4. Make up to 100 $\mu \ell$ with TE buffer.

5. Extract twice with phenol and once with chloroform.

6. Add 1/10th volume of 3 M Na Acetate (pH 5.2.).

7. Add 2.5 volumes of absolute ethanol and place at -70°C for 1 hour.

8. Pellet DNA, rinse with 70% ethanol, absolute ethanol and dry briefly under vacuum.

9. Redissolve in 100 $\mu \ell$ of TE buffer ($\sim$0.1 $\mu g/\mu \ell$).

3.8. CLONING INTO λ PHAGE EMBL3 VECTOR (STRATAGENE)

NOTE:

Microfuge contents of λ arms tube for 2 sec at 13,000 RPM and mix by stirring with micropipette tip before opening.

EMBL3 supplied at 1 $\mu g/\mu \ell$ in TE buffer.

Control insert is PME/BamH I (~12 kbp) at 0.5 μg/ml in TE buffer.

EMBL3 arms plus PME control insert

EMBL3 digested with BamH1 and EcoR I (1.0 μg)	1 $\mu \ell$
(*)PME Insert (0.3 $\mu g/\mu \ell$)	1 $\mu \ell$
10X ligation buffer	1 $\mu \ell$
T4 DNA ligase (4 Weiss units)	1 $\mu \ell$
dH_2O	6 $\mu \ell$

DO NOT EXCEED 5% GLYCEROL (T4 DNA LIGASE IS SUPPLIED IN 50% GLYCEROL)

1. (*)Dilute 3 $\mu \ell$ of PME test insert with 2 $\mu \ell$ of TE Buffer and use 1 $\mu \ell$ (0.3 μg)

2. Incubate at room temperature (22°C) for 1 hour and then at 4°C for 2 days.

TEST:

As above, keeping vector constant (1 μg), and use a range of test insert concentrations (e.g. 0.4, 0.2, 0.1 and 0.0 μg).

Check ligation has been successful by running 1 $\mu \ell$ aliquot on a 0.5% agarose gel comparing with unligated DNA.

3.9. PACKAGING OF LIGATION REACTIONS WITH GIGAPACK II (STRATAGENE)

3.9.1. PREPARATION OF HOST BACTERIA *E. COLI* STRAINS LE392 AND P2392

1. Spread cultures from NZY agar slopes onto NZY agar plates and incubate at 37°C overnight.

2. Store plated strains at 4°C for up to 2 weeks.

3. To prepare plating cultures, pick a single bacterial colony into 40 ml of TB media with 10 mM $MgSO_4$ and 0.2% maltose.

4. Incubate with shaking (200 rpm) at 30°C overnight or with shaking (200 rpm) at 37°C for 4-6 hours. Do not allow growth to exceed $O.D._{600}$ 1.0 /ml as Cells lose their receptors for λ phage attachment if allowed to enter stationary phase.

5. Centrifuge cells at 1000 g for 10 minutes and resuspend pellet (do not vortex) in 0.5 volume of 10 mM $MgSO_4$ (20 ml).

6. Dilute cells to an $O.D._{600}$ of 0.5 with 10 mM $MgSO_4$.

   Use cells immediately or store at 4°C for use within 48 hours. Highest efficiencies are obtained with freshly prepared cells.

   Use 1-4 $\mu\ell$ containing 0.1-5 μg of DNA from the ligation reactions for packaging.

3.9.2. PACKAGING OF LIGATION REACTION

1. Remove appropriate number of extracts from -70°C freezer and place on dry ice. At the same time begin to thaw Sonic extract (yellow tube).

2. Quickly thaw Freeze/Thaw extract (red tube) between fingers until just melted.

3. Add DNA (1-4 $\mu\ell$) **IMMEDIATELY** to Freeze/Thaw extract (red tube).

4. Quickly add 15 $\mu\ell$ of Sonic extract (yellow tube).

5. Microfuge for 2 sec and mix well by stirring with a micropipette tip.

6. Incubate at room temperature (22°C) for 2-3 h.

7. Add 500 $\mu\ell$ of λ SM buffer.

8. Add 20 $\mu\ell$ of chloroform. Mix gently.

9. Microfuge for 10 seconds to sediment debris. Store supernatant at 4°C (do not freeze) if not to be titred immediately.

10. Supernatant is now ready to be titred.

For a 100 mm plate, prepare 10-fold dilutions to 10^{-4} of supernatant in λ phage dilution buffer (final volume 10 $\mu\ell$).

Add 200 $\mu\ell$ of plating cells and incubate at 37°C for 15-20 minutes with gentle mixing.

Add 3 ml of Top agar cooled to 55°C and pour over a dry, prewarmed (37°C) NZY agar plate and incubate at 37°C overnight.

Control: Test efficiency of packaging with wild type cI857 Sam7.

1. Resuspend wild type DNA (cI857 Sam7) overnight in 10 $\mu\ell$ of TE buffer, pH 7.4.

2. Use 1 $\mu\ell$ (~0.2 μg of DNA) and proceed with packaging as described above.

3. Make two consecutive 10^{-2} dilutions of the λ phage packaged supernatant in λ phage dilution buffer (the final is a 10^{-4} dilution).

4. Plate 10 $\mu\ell$ of the 10^{-4} dilution on the host strain VSC257 and incubate for at least 12 hours at 37°C.

5. Count number of plaques. There should be ~400 plaques on the 10^{-4} dilution plate.

The efficiency of packaging in the construction of the λ phage library is calculated by:

$$\frac{(\text{Nº of plaques}) \times (\text{dilution factor}) \times (\text{total packaging volume } 500\ \mu\ell)}{(\text{amount DNA [in } \mu\text{g] packaged}) \times (\text{Nº of } \mu\ell \text{ plated})}$$

3.10. AMPLIFICATION AND SCREENING OF LIBRARY

1. Prepare two large (24 x 24 cm) NZY agar plates (about 330 ml per plate).

2. Dry plates overnight at 37°C or for 2 hours under an operating Laminar Flow hood with the lids removed.

3a. Make 100 $\mu\ell$ dilution of packaged DNA in λ phage dilution buffer to give ~10^5 λ phage particles for plaque screening (as defined from initial titration experiments).

3b. Add 1 μg of packaged DNA to λ phage dilution buffer (final volume 100 $\mu\ell$) for library amplification.

4. Add each aliquot to 3 ml of freshly prepared P2392 plating cells.

5. Mix by inversion and incubate at 37°C for 15 minutes with gentle shaking..

6. Add to 45 ml of top agar cooled to 55°C in a 50 ml polypropylene centrifuge tube.

7. Mix rapidly by inverting the tube 3-4 times and pour immediately over a prewarmed (37°C) NZY plate starting from one corner. Tilt plate to spread agarose.

8. Allow to solidify and incubate at 37°C overnight.

3.10.1. AMPLIFYING LIBRARY

1. Flood a plate with 50 ml of λ phage dilution buffer and place at 4°C overnight (with gentle rocking if possible).

2. Pour the fluid into two sterile 50 ml polypropylene centrifuge tube. Rinse plate with 20 ml of λ phage dilution buffer and add to the centrifuge tubes.

3. Add chloroform to 5% of total volume in each tube and leave at room temperature for 15 minutes.

4. Centrifuge at 2000 x g for 5 minutes.

5. Recover the supernatants to a sterile polypropylene or glass bottle. Add chloroform to 0.3% and store in aliquots at 4°C.

   Do not store library below 4°C.

6. Check titre of the library on freshly prepared P2392 plating cells which should be about 10^{10} pfu/ml.

7. For long term storage of amplified library, add 70 $\mu\ell$ of 100% DMSO to 1 ml aliquots and store at -70°C.

3.10.2. SCREENING OF LIBRARY

1. Cool plate for 2 hours at 4°C after the overnight incubation to harden the top agar.

2. Place a sheet of dry nylon membrane 20 cm x 20 cm (Hybond-N) on the lawn of cells ensuring no air bubbles are trapped.

3. Allow absorbtion of λ phage particles to occur for 3 minutes.

4. During the absorbtion pierce several holes of different patterns through the membrane and into the gel at the top four corners of the plate using a hypodermic needle. This will enable the subsequent alignment of the autoradiograph from the filter with the library plate.

5. If required, repeat steps 2-4 with a second and third membrane allowing absorption to occur for 4 and 5 minutes respectively. Position the membrane over the same area as the first. An impression of the holes pierced into the gel should be just visible. Carefully mark the position of the holes on the fresh membranes by piercing lightly with a hypodermic needle.

6. Carefully peel filter from the plate using forceps. Avoid removing any top agar.

7. Place membrane, <u>plaque side up</u>, on three sheets of Wattman 3M filter paper soaked in denaturing solution. Leave for 7 minutes.

8. Transfer membrane to a second set of filters soaked in neutralising solution. Leave for 3 minutes.

9. Rinse filter in 2X SSC.

10. Leave filter to dry at room temperature or 37°C. Cover in Saran® Wrap and place **plaque side down** on a standard UV transilluminator for 3 minutes.

DENATURING SOLUTION

NaOH	0.5 M
NaCl	1.5 M

NEUTRALISING SOLUTION

Tris-HCl, pH 7.2	0.5 M
NaCl	1.5 M
EDTA (Na_2)	0.001 M

3.11. OLIGONUCLEOTIDE LABELLING OF DNA

The concentration of DNA required for labelling is usually 10-100 ng and should be contained in a volume of ~3-5 $\mu\ell$. DNA can be labelled in the presence of agarose.

1. Place gel in screw-capped tube and immerse in boiling water bath for 7 minutes. Transfer to 37°C water bath for 10 minutes.

2. Add the following in order:

Oligolabelling buffer	3 $\mu\ell$
Bovine serum albumin (10 mg /ml)	0.6 $\mu\ell$
DNA	10 ng
dCTP 5'-[α-^{32}P] triphosphate (10 uCi/$\mu\ell$)	1.5 $\mu\ell$
DNA polymerase Klenow fragment (1 unit /$\mu\ell$)	0.6 $\mu\ell$

   Make up to 15 $\mu\ell$ with dH_2O

3. Allow reaction to proceed at room temperature for 5 hours to overnight or at 37°C for 2 hours.

4. Place in boiling water bath for 10 minutes and cool rapidly on ice for 3 minutes.

3.12. HYBRIDISATION USING BSA/SDS[8]

1. Rinse membrane with 5X SSC and prehybridise for 4 hours to overnight at 65°C using approximately 20 ml of prewarmed hybridisation solution.

2. Pour off and replace with fresh prewarmed hybridisation solution.

3. Add the probe at 1-10 x 10^6 cpm/ml and hybridise for 18 hours at 65°C.

4. Wash membrane in 2X SSC-0.1% SDS for 15 minutes at room temperature.

5. Repeat step 4.

6. Wash the filter in 2X SSC-0.1% SDS for 15 minutes at 65°C.

7. Wash filter in 1X SSC for 30 minutes at 65°C.

7a. For DNA dot-blots, wash filter in 0.1X SSC for 30 minutes at 65°C.

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[8] The method is based on the hybridisation solution described by Church and Gilbert (Church, G. M. and Gilbert, W. (1984). Genomic sequencing. *Proceedings of the National Academy for Science (USA).* **81:** 1991-1995) but differs in the use of SSC and SDS in the washing stages as recommended by Westneat, D.F., Noon, W.A., Reeve, H.K. and Aquadro, C.F. (1988). Improved hybridisation conditions for DNA 'fingerprints' probed with M13. *Nucleic Acids Research.* **16**: 4161.

3.13. PLAQUE PURIFICATION

1. Pick a plug of agar containing a single λ phage plaque using a glass pasteur pipette and blow into 1 ml of λ phage dilution buffer.

2. Incubate at room temperature for 1 hour or 15 minutes at 37°C. The titre of the λ phage will be approximately 10^6 pfu/ ml.

3. It is desirable to have ~100 pfu on a 94 mm diameter petri dish. Add 10 $\mu\ell$ of the original stock solution from step 2 to 1 ml of λ phage dilution buffer and use 10 $\mu\ell$ of this for plating.

4. Add 10 $\mu\ell$ of diluted λ phage to 200 $\mu\ell$ of host bacteria and incubate at 37°C for 10 minutes.

5. Add 3 ml of top agar cooled to 55°C and pour over a 94 mm diameter NZY agar plate that has been prewarmed to 37°C. Allow to set and incubate at 37°C overnight.

3.14. ISOLATION OF λ PHAGE DNA

Prepare *E. coli* plating cells of strain LE 392 as described above. For a 140 mm diameter petri dish use 0.5 ml of plating cells and 7.5 ml of Top agar. For this size of plate confluent lysis is reached with ~10^5 λ phage particles.

1. Pick a single λ phage plaque into 1 ml of λ phage dilution buffer and incubate for 15 minutes at 37°C or 1 hour at room temperature (~10^6 pfu/ml).

2. Add 100 $\mu\ell$ of phage to 500 $\mu\ell$ of plating cells. Incubate at 37°C for 15 minutes.

3. Add 7.5 ml of top agar cooled to 55°C and pour evenly over a NZY agar plate. Incubate at 37°C overnight. This should result in an almost complete lysis of the *E. coli* lawn.

4. Flood the plate with 15 ml of λ phage dilution buffer and place at 4°C for 2 hours to overnight.

5. Pour into a 50 ml polypropylene centrifuge tube and centrifuge at 3000 rpm for 5 minutes.

6. Transfer supernatant to a fresh tube and add:

RNase A (5 mg/ml) . . . . . 3 $\mu\ell$ (1 μg/ml)
DNase I (5 mg/ml) . . . . . 3 $\mu\ell$ (1 μg/ml)

Incubate at 37°C for 15 minutes in a water bath.

7. Add an equal volume of 20% PEG 8000 : 12% NaCl. Vortex well and place on ice for 2 hours to overnight.

9. Centrifuge at 3000 rpm for 30 minutes and remove all the supernatant. Recentrifuge to assist.

10. Resuspend by pellet in 1 ml of:

Tris-HCl (pH 8.0) . . . . . 50 mM
$MgCl_2$ . . . . . 10 mM

Vortex until dissolved and transfer to a microcentrifuge tube.

11. Phenol and chloroform extract once each.

12. Add 1/10th volume of 3 M Na acetate (pH 5.2.) and 1 volume of iso propanol. Vortex and leave at room temperature for 1 hour. Microfuge at 12000 rpm for 10 minutes at room temperature.

13. Wash pellet with 1 ml of 70% ethanol, 1 ml of absolute ethanol and desiccate.

14. Dissolve pellet in 200 $\mu\ell$ of TE buffer (pH 8.0).

Samples are rich in RNA therefore include RNase A (1 $\mu\ell$ of 5 mg/ml) in all restriction endonuclease digests.

Heat samples at 65°C for 5 minutes and place on ice following restriction endonuclease digestion to prevent annealing between the λ arm cos ends.

3.15. RECOVERY OF DNA FROM AGAROSE GELS WITH SILICA POWDER[9]

(Used to purify DNA fragments in solution or from TAE agarose gels)

1. Dissolve agarose or DNA in solution in 3 volumes (w/v for DNA in agarose) of NaI solution for 5-10 minutes at 50°C with occasional mixing.

2. Shake glass powder into suspension. Add 5 $\mu\ell$ /μg of DNA to the dissolved gel and incubate on ice for 10 minutes with occasional mixing to resuspend the glass slurry.

3. Microfuge at 12,000 rpm for 10 seconds and remove supernatant with a small bore pipette. Recentrifuge to assist. Wash pellet with 500 $\mu\ell$ of NaI solution by resuspending glass slurry with a pipette. Microfuge and remove all supernatant.

4. Gently wash pellet three times with 500 $\mu\ell$ of cold ethanol wash solution. Microfuge at 12,000 rpm for 10 seconds and remove all supernatant between washes. The final pellet may be dried briefly under vacuum to remove all traces of ethanol from the glass.

5. Elute DNA by resuspending the pellet in 10-20 $\mu\ell$ TE buffer or sterile H_2O and incubate at 50°C for 5-10 minutes with occasional mixing.

   Microfuge at 12,000 rpm for 1 minute and retain the supernatant containing the purified DNA.

SILICA GLASS SLURRY

Resuspend 80 g of powder (Sigma) in 160 ml of dH_2O and stir for one hour at room temperature. Leave to settle for 90 minutes in a measuring cylinder. Take **SUPERNATANT** and centrifuge at 6000 rpm for 10 minutes. Resuspend pellet in 30 ml of dH_2O. Add nitric acid to 50% (43 ml of 70% acid) and heat to 98°C in a fume cupboard. Allow to cool, recentrifuge as described above and wash pellet 4-5 times with dH_2O until the pH returns to neutral. Resuspend final pellet as a 50% slurry in dH_2O. Aliquot and autoclave at 121°C for 15 minutes. Store at 4°C.

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[9] Although the method is designed for use with TAE gels, it may work with those prepared in TBE if Na-phosphate, pH 6.5 is added to 100 mM final concentration before addition of the NaI and incubation. The method works most efficiently for DNA <20 kbp and >100 bp for which approximately 70-80% recovery is possible.

3.16. DNA LIGATION WITH pUC PLASMID VECTORS

Reaction Mix

Vector DNA	100 ng
*DNA fragment	10 ng
5X ligation buffer	2 $\mu\ell$
T4 DNA ligase	4 U
dH_2O	to 10 $\mu\ell$

*Keep vector constant but vary inset ration using 10, 1, 0.1 ng/ml.

Microfuge briefly and incubate at room temperature for 2 hours followed by overnight at 16°C.

Heat at 65°C for 10 minutes.[10]

Chill on ice and store at -20°

Use 1-2 $\mu\ell$ (10-20 ng of DNA) of the ligation reaction to transform competent *E. coli*.

Controls

Phosphatase treated vector DNA.

Restriction endonuclease digested insert DNA.

Known concentration of intact vector DNA to measure the transformation efficiency.

Selection of transformants

Add to a petri dish 40 $\mu\ell$ of 50 mg/ml ampicillin and add 20 ml of LB agar cooled to 50°C. Mix thoroughly by swirling and allow to set. The final concentration of ampicillin is 100 μg/ml.

Spread over the surface of the LB agar plate:

X-gal (20 mg/ml in dimethylformamide)	40 $\mu\ell$
IPTG (10 mg/ml in dH_2O)	40 $\mu\ell$

Dry plates at 37°C. Store at 4°C for use within 48 hours.

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[10] Heating at 65°C for 10 minutes at the end of the ligation reaction reduces the number of background non-recombinants to zero. Nicholls, R.D. (1986). Direct cloning in plasmid libraries after fragment enrichment. *Focus (Bethesda Research Laboratories)*. **8**: 1-3.

3.17. SIMPLE TRANSFORMATION OF *E. COLI* [11]

1. Inoculate 10 ml of LB broth in a 50 ml centrifuge tube with a single colony of *E.coli*. Incubate overnight at 37°C in a shaking water bath at 150 rpm.

2. Add 0.5 ml of culture to 50 ml of prewarmed LB broth in a 150 ml centrifuge tube. Incubate at 37°C with shaking at 175 rpm until the $O.D_{600}$ is 0.3-0.4 (~90 minutes).

3. Remove 10 ml of culture and add an equal volume of 2X TSS Buffer. Mix thoroughly and place in ice.

3a. For long-term storage, the cells are flash-frozen in a dry ice/ethanol bath and stored at -70°C.

3b. Frozen competent cells are thawed slowly on ice and used immediately.

4. For transformation, a 0.1 ml aliquot of cells is pipetted into a cold polypropylene tube containing 1 $\mu\ell$ (~10 pg-1 ng) of plasmid DNA and mixed gently.

5. Stand in ice for 30 minutes.

6. Add 0.9 ml of LB Broth containing 20 mM glucose and incubate at 37°C for 1 hour with shaking at 225 rpm.

7. Select transformants by appropriate methods.

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[11] Chung, C.T., Niemela, S.L. and Miller, R.H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Science (USA)*. **86**: 2172-2175.

3.18. ISOLATION OF PLASMID DNA[12]

1. Inoculate 5 ml of LB medium with a single bacterial colony and incubate overnight at 37°C with shaking at 225 rpm.

2. Add 1.5 ml into microfuge tube and centrifuge at 12,000 rpm for 1 minute at 4°C. Remove all the supernatant leaving the pellet as dry as possible.

3. Add 2 $\mu\ell$ of 5 mg/ml RNase A.

4. Add 200 $\mu\ell$ of ice cold GET and resuspend pellet by vortexing ensuring all the cells are in suspension. Place on ice for 5 minutes.

5. Add 400 $\mu\ell$ of freshly prepared Alkaline-SDS. Mix gently by inversion two-three times and place on ice for 5 minutes.

6. Add 300 $\mu\ell$ of ice cold 3 M KAc, pH 4.8. Invert tube and vortex 10 seconds. Place on ice for 5 minutes.

7. Centrifuge at 12,000 rpm for 15 minutes at 4°C and transfer supernatant to fresh tube.

8. Add an equal volume of phenol-chloroform. Vortex and centrifuge at 12,000 rpm for 5 minutes at room temperature. Transfer aqueous phase to fresh tube.

9. Add and equal volume of isopropanol, vortex and leave at room temperature for 5 minutes.

10. Centrifuge at 12,000 rpm for 15 minutes and carefully remove all supernatant (recentrifuge to assist).

11. Wash pellet twice with 500 $\mu\ell$ of 70% ethanol and dry pellet briefly in vacuum desiccator.

12. Redissolve pellet in 50 $\mu\ell$ of dH_2O.

Use 5 $\mu\ell$ of the sample for restriction enzyme digestion in a volume of 20 $\mu\ell$.

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[12] Modification in Sambrook *et al,* (1989). *Molecular cloning: a laboratory manual* (second edition). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. of the method of Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research.* **7**: 1513.

3.19. ALKALINE SOUTHERN TRANSFER OF DNA

1. Soak gel in 3-4 gel volumes of 0.25 M HCl for until bromophenol blue tracking dye turns yellow and leave for a further 10 minutes.

   This step is necessary only for DNA fragments >10 kb.

2. Pour off HCl and rinse gel in dH_2O.

3. Place in 3-4 gel volumes of denaturing solution for 30 minutes with gentle agitation.

4. Place a 1" high stack of towels, cut slightly larger than the gel, into a developing tray and saturate with denaturing solution.

5. Cover with a sheet of thick blotting paper and place gel on top. Remove air-bubbles from under the gel by smoothing with a gloved hand.

6. Edge the gel with strips of Saran® Wrap to prevent direct contact between the towels and the nylon membrane.

7. Cut nylon membrane (Hybond N or N^+) slightly smaller than the gel and place carefully on to the gel avoiding air bubbles.

8. Cover with three sheets of thick blotting paper soaked in denaturing solution.

9. Place a 3" high stack of paper towels on top and cover with a flat plate (~200 g).

10. After 1 hour remove bottom layer of towels that have absorbed the denaturing solution.

11. Allow transfer to continue for a further 6 hours to overnight.

12. Carefully remove membrane and rinse in neutralising solution followed by 2X SSC.

13. Air dry membrane at room temperature and place DNA side face down on transilluminator for 3 minutes. Seal in Saran® Wrap and store at -20°C.

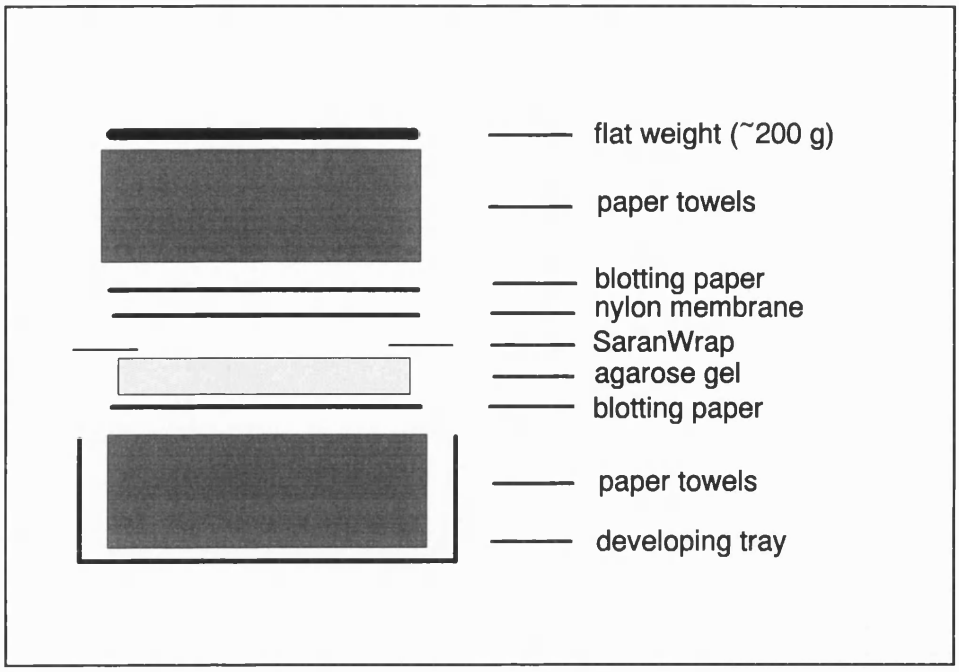

Figure 81 Alkaline transfer of DNA from an agarose gel to a nylon membrane

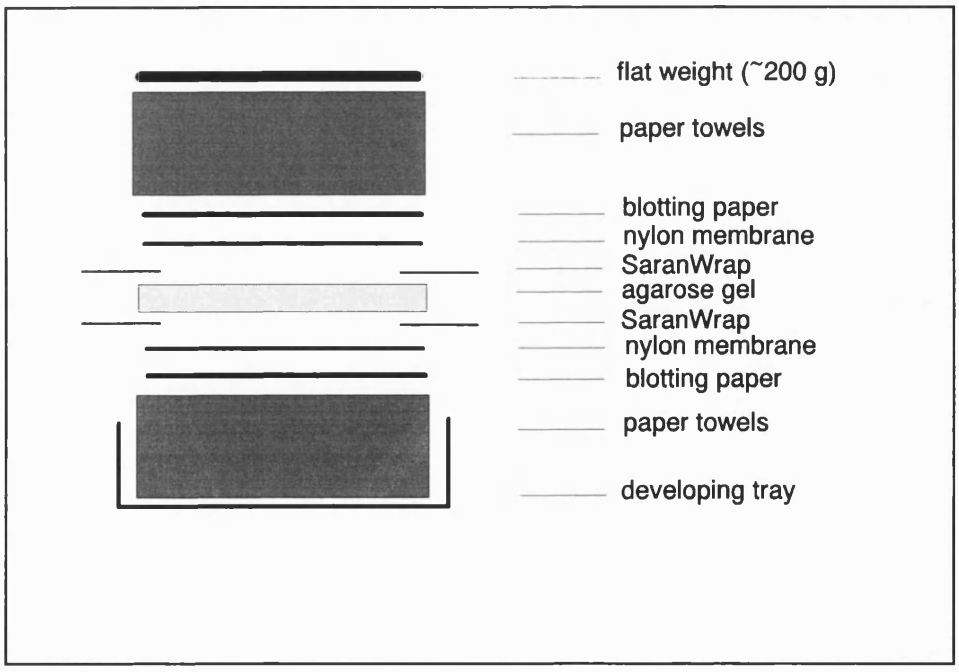

Figure 82 Alkaline transfer of DNA from an agarose gel to duplicate nylon membranes

3.20. DNA DOT-BLOTTING

A. DNA samples

1. Prepare DNA samples in 200 μl of TE buffer.[13]

2. Add an equal volume of 0.8 N NaOH-20 mM EDTA and vortex.

3. Place in boiling water bath for 10 minutes and chill on ice.

4. Wet nylon membrane (9 x 12 cm sheet) in dH_2O. Blot excess water, place in dot blot apparatus and tighten seal under vacuum.

5. Load samples in to wells and allow to pass slowly (>5 minutes) through the membrane under slight vacuum. With a Nalgene hand pump, a 1/4 squeeze of the handle gives a flow rate of ~200 $\mu\ell$/5 minutes.

6. Either seal unused wells with tape or fill with TE buffer.

7. Rinse wells with 0.4 N NaOH-10 mM EDTA under vacuum, allowing membrane to dry.

8. Remove membrane and rinse twice in 200 ml of 2X SSC.

9. Air dry, cover in Saran® Wrap and UV irradiate DNA side down for 3 minutes.

B. Environmental samples

1. Add 200 $\mu\ell$ of 0.4 N NaOH-10 mM EDTA to wells of 96 place round bottom microtitre plate.

2. Using a 1 $\mu\ell$ disposable plastic loop, sweep approximately 2 cm of the leading edge of trophozoite growth on a NNA-*K10896* plate and inoculate into well of a microtitre plate.

3. Seal plate, incubate at 70°C for 15 minutes and chill on ice.

4. Proceed with steps 4-9 above.

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[13] The alkaline-heat denaturation of the DNA can also be performed in a 96 place microtitre plate using 100 μl of DNA sample per well. After adding an equal volume of 0.8 N NaOH-20 mM EDTA, the plate is sealed with sticky tape and a few holes made in the tape over the wells with a needle. The plate is then floated in a water bath at 70°C for 15 minutes and placed in a -20°C freezer for 4 minutes.

3.21. DNA SEQUENCING WITH SEQUENASE® VERSION 2.0 (UNITED STATES BIOCHEMICAL)

3.21.1. PREPARATION OF PLASMID DNA TEMPLATE

A. Preparation of Sephadex G-50 spin column

1. Add 10 g of Sephadex G-50 powder to 250 ml of dH_2O and leave to swell for 3 hours.

2. Using a buchner funnel, wash gel with 1 L of dH_2O. Do not allow gel to dry.

   (extensive washing of the Sephadex gel is important to remove soluble dextran which can inhibit some reactions)

3. Autoclave the gel slurry (~100 ml) at 121°C for 15 minutes.

4. Pierce the end of a sterile 0.5 ml eppendorf tube with a red-hot hypodermic needle.

5. Plug the tube with a small amount of sterile, siliconized glass wool.

6. Place the plugged tube into a sterile 1.5 ml eppendorf tube.

7. Add 200 $\mu\ell$ of gel to the plugged tube and centrifuge at 2000 rpm in a swing-out rotor for 3 minutes.

8. Add 200 $\mu\ell$ of TE buffer (pH 7.5) to the column and centrifuge as above.

9. Transfer column to a fresh microcentrifuge tube.

B. Alkaline denaturation of plasmid DNA

1. Take 18 $\mu\ell$ of purified plasmid DNA and add 2 $\mu\ell$ of 2 M NaOH. Centrifuge at 12000 rpm for 2 seconds. Mix thoroughly and recentrifuge. Leave at room temperature for 5 minutes.

2. Add denatured DNA to the Sephadex G-50 spin column and centrifuge in a swing-out rotor at 2000 rpm for 3 minutes.

   Denatured plasmid DNA equilibrated in TE buffer is contained in the lower microcentrifuge tube.

3.21.2. DNA SEQUENCING WITH SEQUENASE® VERSION 2.0

1. ANNEALING TEMPLATE AND PRIMER

Primer . . . . . 1 $\mu\ell$
Reaction buffer . . . . . 2 $\mu\ell$
Denatured DNA template . . . . . 7 $\mu\ell$

Place in water at 65°C and leave to cool to <30°C in about 30 minutes. Use within 4 hours.

Whilst primer is annealing prepare:

i. **Labelling Mix** (step 2a)

ii. **Termination Mixes** (step 3) in a microtitre plate

2. LABELLING REACTION

a. Dilute Labelling Mix (dGTP, green-capped tube) 1:5 with dH_2O (eg. 4 $\mu\ell$ of Mix + 16 $\mu\ell$ of water).

b. Dilute Sequenase enzyme 1:8 in ice-cold Enzyme Dilution Buffer. Store on ice and use immediately.

c. Template-Primer (step 1) . . . . . 10 $\mu\ell$
DTT (0.1 M) . . . . . 1 $\mu\ell$
Dilute Labelling Mix (step 2a) . . . . . 2 $\mu\ell$
α-^{35}S]dATP . . . . . 0.5 $\mu\ell$
Diluted Sequenase . . . . . 2 $\mu\ell$

ALWAYS ADD THE ENZYME LAST

Mix thoroughly (avoiding bubbles) and incubate at room temperature for 2-5 minutes.

3. TERMINATION TUBES

Label four tubes G, A, T, C

Add 2.5 $\mu\ell$ of the Termination Mixes ddGTP, ddATP, ddTTP and ddCTP (red-capped tubes) to corresponding tubes labelled G, A, T, C. Cap the tubes and warm to 37°C for at least 1 minute

4. TERMINATION REACTION

a. When the Labelling reaction is complete (step 2), remove 3.5 $\mu\ell$ and add to the Termination tube labelled G. Mix, centrifuge and place at 37°C. Similarly, repeat for Termination tubes A, T and C.

b. Incubate at 37°C for 3-5 minutes (times of up to 30 minutes should not effect results)

c. Add 4 $\mu\ell$ of Stop Solution to each Termination reaction. Mix thoroughly.

d. Store sequencing reactions on ice for immediate use or freeze at -20°C for use within 1 week.

e. Heat samples to 80°C for 2 minutes and load 2-3 $\mu\ell$ immediately on to a sequencing gel.

3.22. PREPARATION OF DNA SEQUENCING GELS

1. Take two glass plates, one larger than the other. Wash both with detergent, rinse well with water, polish with ethanol and air dry.

2. Take the **SMALLER** of the two plates and wipe with a tissue soaked in dimethyldichlorosilane to render it non-stick. Rinse with water and air dry.

3. Take the **LARGER** plate and treat with Silane A to enable the gel to adhere:

Silane A-174 (BDH)	0.2 ml
Acetic acid (10%)	0.6 ml
Absolute ethanol	20 ml

Pour over entire surface of gel and leave for 2 minutes. Rinse with water and air dry.

6% POLYACRYLAMIDE SEQUENCING GEL

4. SequaGel™ concentrate . . . . . 12 ml
   SequaGel™ diluent . . . . . 33 ml
   SequaGel™ buffer . . . . . 5 ml

   Mix gently, avoiding air bubbles and add:

   Ammonium persulphate (16 g/100 ml in dH_2O) . . . . . 300 $\mu\ell$
   TEMED (ultrapure, BRL) . . . . . 30 $\mu\ell$

5. Mix gently, avoiding air bubbles and use immediately. Allow gel to polymerise for 1 hour to overnight.

6. Rinse wells of gel with 1X TBE buffer to remove urea.

7. Run gel in 1X TBE at 30 mA for 30 minutes.

8. Rinse wells again, load samples (2-3 $\mu\ell$). Run gel at 30 mA for 2-4 hours and at a temperature of 55-60°C.

9. Lay gel flat and gently prise away upper plate.

10. Soak gel in 10% methanol-10% acetic acid for 30 minutes to cross-link the polyacrylamide and prevent diffusion of the DNA bands.

11. Rinse gently in dH_2O for 30 minutes to remove the urea from the gel.

12. Dry in a hot air oven at 80°C for 30 minutes.

13. Cover gel with X-ray film and expose for 18-36 hours.

14. Develop autoradiograph and record DNA sequence.

3.23. PURIFICATION OF OLIGONUCLEOTIDE PRIMERS

1. Attach a 2 ml luer fitting syringe to the column.

2. In a fume cupboard, draw 1 ml of concentrated ammonia solution into a second 2 ml syringe and attach to the other end of the column.

3. Pass 0.2 ml of the ammonia solution through the column into the empty syringe and leave at room temperature for 20 minutes.

4. Repeat step 3 twice more and finally pass the remainder of the ammonia solution through the column and leave for 20 minutes.

5. Pass the ammonia solution back and forth through the column between the two syringes several times.

6. Add the ammonia containing the eluted oligonucleotides to a 2 ml screw capped eppendorf tube and incubate overnight at 55°C.

7. Chill tube on ice for 5 minutes and add to a 15 ml polypropylene tube containing 1 ml of ice cold glacial acetic acid and 6 ml of absolute ethanol.

8. Mix well and place at -20°C for 1 hour.

9. Centrifuge at 3000 rpm for 20 minutes.

10. Carefully remove all the supernatant by aspiration and wash pellet twice with 3 ml of 80% ethanol. Remove supernatant and dry pellet at 37°C for 30 minutes.

11. Dissolve pellet in 500 $\mu\ell$ of ddH_2O with warming at 37°C for 1 hour.

12. Dilute 10 $\mu\ell$ of the supernatant with 1 ml of ddH_2O and scan in a quartz cuvette from 300 nm to 240 nm. A clear, sharp peak should be observed around 260 nm.

13. Taking into account the dilution factor of 1:100, estimate the concentration of the original oligonucleotide solution assuming 1 OD_{260} is equivalent to 37 μg/ml of oligonucleotide present.

3.24. PCR "MASTER MIX" CALCULATIONS

PCR MASTER MIX															
VOLUMES IN $\mu\ell$	**X1**	**X2**	**X3**	**X4**	**X5**	**X6**	**X7**	**X8**	**X9**	**X10**	**X11**	**X12**	**X13**	**X14**	**X15**
X10 TAQ BUFFER	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
dATP (10 mM)	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
dCTP (10 mM)	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
dGTP (10 mM)	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
dTTP (10 mM)	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
PRIMER 1 (10 μM)	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
PRIMER 2 (10 μM)	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
ddH_2O	41.5	83	124.5	166	207.5	249	290.5	332	373.5	415	465.5	498	539.5	581	622.5
TAQ (5U/$\mu\ell$)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5
TOTAL VOLUME	80	160	240	320	400	480	560	640	720	800	880	960	1040	1120	1200
ADD 80 $\mu\ell$ OF MASTER MIX PER TUBE CONTAINING 20 $\mu\ell$ OF DNA IN ddH_2O															

3.24.1. PERFORMING THE POLYMERASE CHAIN REACTION

1. Take 100-200 ng of DNA and make up to 20 $\mu\ell$ with ddH_2O in a 0.5 ml eppendorf tube.

2. Add 75 $\mu\ell$ of mineral oil and microfuge for 2 seconds

3. Place tube in thermal cycler and heat to 95°C for 5 minutes and chill tubes on ice.

4. Prepare reaction Master Mix and place on ice.

5. Add 80 $\mu\ell$ of Master Mix to the DNA tube, microfuge for 2 seconds, mix and spin tubes again.

6. Start thermal cycler. Add tubes to the machine when the temperature reaches 85°C on the first cycle.

7. For primer set pB2.3, for the amplification of *N. fowleri* DNA, perform **35 cycles** of:

   Step 1 . . . . . 95°C for 1 minute

   Step 2 . . . . . 55°C for 1 minute

   Step 3 . . . . . 72°C for 1.5 minute

   Hold tubes at 72°C for 7 minutes after step 3 of the final cycle.

8. Place samples at -70°C for 15 minutes and remove upper oil layer. Analyze 10-20 $\mu\ell$ aliquots on a 1.2% agarose-TBE gel at 80 volts for 2 hours. Include 0.5 μg/ml ethidium bromide in the gel and running buffer.

3.25. RAPID EXTRACTION OF DNA FROM FREE-LIVING AMOEBA FOR PCR

3.25.1. METHOD A: NNA-*K. EDWARDSII* CULTURES

1a. With a 1 $\mu\ell$ disposable inoculating loop, gently scrape a 1 cm area of trophozoites or cysts from the culture plate. Avoid collecting any agar.

2a. Suspend cells in a 0.5 ml eppendorf tube containing: 10 $\mu\ell$ of X10 Taq buffer + 1 $\mu\ell$ of 10 mg/ml proteinase K + 59 $\mu\ell$ of dH_2O.

Lysis mixture for **10 samples** would be:

X10 Taq Buffer . . . . . 100 $\mu\ell$
Proteinase K (10 mg/ml) . . . . . 10 $\mu\ell$
dH_2O . . . . . 590 $\mu\ell$

Distribute in 70 $\mu\ell$ aliquots per tube.

3a. Add two drops of sterile mineral oil from a 1 ml plastic paster pipette.

4a. Incubate at 60°C in a water bath for 1 hour.

5a. Place in boiling water bath for 10 minutes and chill on ice.

3.25.2. METHOD B: LIQUID CULTURES

1b. Transfer 1 x 10^3-1 x 10^4 cells to a 0.5 ml eppendorf tube. Wash the cells once with phosphate buffered saline by centrifugation at ~6,500 g in a microcentrifuge for 10 seconds.

2b. Resuspend cells in 20 $\mu\ell$ of dH_2O.

3b. Add 10 $\mu\ell$ of X10 Taq buffer + 1 $\mu\ell$ of 10 mg/ml proteinase K + 39 $\mu\ell$ of dH_2O and mix.

Lysis mixture for **10 samples** would be:

X10 Taq Buffer . . . . . 100 $\mu\ell$
Proteinase K (10 mg/ml) . . . . . 10 $\mu\ell$
dH_2O . . . . . 390 $\mu\ell$

4b. Add two drops of sterile mineral oil from a 1 ml plastic paster pipette.

5b. Incubate at 60°C in a water bath for 1 hour.

6b. Place in boiling water bath for 10 minutes and chill on ice.

7. To each tube add:

dNTP's (10 mM each)	2 $\mu\ell$ each
primer 1 (10 μM)	10 $\mu\ell$
primer 2 (10 μM)	10 $\mu\ell$
dH_2O	1.5 $\mu\ell$
Taq polymerase (5 U/$\mu\ell$)	0.5 $\mu\ell$

A X10 "Master Mix" would be:

dNTP's (10 mM each)	20 $\mu\ell$ each
primer 1 (10 μM)	100 $\mu\ell$
primer 2 (10 μM)	100 $\mu\ell$
dH_2O	15 $\mu\ell$
Taq polymerase (5 U/$\mu\ell$)	5 $\mu\ell$

8. Microfuge briefly

9. Perform PCR:

i 95°C	1 minute
ii 55°C	1 minute
iii 72°C	1.5 minutes

- 35 cycles -

iv 72°C	7 minutes